

LIPOLYTIC ACTIVITY OF BACTERIA:

A STUDY

OF DIFFUSIBLE LIPASES PRODUCED

BY BACTERIA

WITH PARTICULAR REFERENCE TO

STAPHYLOCOCCAL LIPASE

IN

CULTURE FILTRATES

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INTRODUCTION

Interest was first aroused concerning the action of micro-organisms, notably bacteria, on fats soon after bacteriology became an established branch of science. Observations were made by Escherisch (1886), who demonstrated that bacteria could act on neutral fats, splitting the fat molecule into free fatty-acids and glycerol. In the same year, Nencki (1886) showed by experiments in vitro and in vivo that some bacteria are very active in the utilisation of fats, but that they do not play any significant part in the breakdown of fats in the intestine of animals. It was, however, subsequently found that even in cases of disease of the pancreas, where lipase secretion is greatly reduced, a small but definite proportion of fat ingested is split in the intestine, and this splitting has been attributed to the activity of intestinal bacteria; Stewart and Dunlop (1937).

Nencki's observations, made at the very commencement of studies in this field, had an important directive influence on subsequent work. Literature published during the next sixty years shows that almost all investigations in this field have been confined to saprophytic and commensal micro-organisms, such as are concerned with food-spoilage and the deterioration of

commercial products, in which processes micro-organisms play a part equal in importance to that of light, atmospheric oxygen, metallic catalysts and electromagnetic rays; Jensen and Grettie (1937).

A few investigators have recorded work endeavouring to associate lipolysis with other characters such as haemolysis, associated with pathogenicity, but results of different workers tend to be contradictory, and the reader has the impression that, in general, knowledge of the practical importance of bacterial lipolysis is unsatisfactory and confused.

In the work to be recorded the first necessity was to compare the different techniques for demonstrating lipolysis which had previously been described by various workers . Then, using the optimal method, and with such modifications in the medium as were necessary to support the growth of delicately growing organisms, a study was made of as wide a range of bacterial species as possible for the production of diffusible lipase. It was further hoped to demonstrate lipase in culture filtrates of species giving positive results, and to study some of the characteristics of these filtrable lipases.

During the course of the work several types of media were developed which were suitable for demonstrating bacterial lipolysis in different circumstances, but only a small number of species were found to produce diffusible

lipases, the Staphylococci being the only group from which significant positive results were obtained. It was, therefore, apparent that further work would have to be confined to this genus.

When no definite relationship could be established between lipolysis and characteristics associated with pathogenicity in the Staphylococci, an attempt was made to correlate lipolysis with the saprophytic mode of living in this and some other genera. The results of this investigation were largely in agreement with those published by other workers.

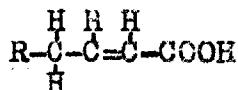
The final sections of this work were the production of lipolytic filtrates from cultures of Staphylococci, the production of antilipase in rabbits by immunisation with these filtrates, and a study of the immunological specificity of staphylococcal lipase.

Brief descriptions are included in this record of some properties of natural fats, to show the nature of substrates provided for bacteria by the lipids, and of the general properties of enzymes, and are followed by a short account of the theory of bacterial lipolysis as it has been evolved by various research workers.

SOME PROPERTIES OF NATURAL FATS

Natural fats are esters of the higher alcohols with saturated and unsaturated aliphatic monocarboxylic acids. There are three types - oils, fats and waxes - grouped together under the term 'lipids' or 'Neutral fats'. By far the commonest alcohol occurring in lipids is the trihydric alcohol glycerol, $C_3H_8O_3$, but other alcohols such as the sterols occur frequently, particularly in waxes. (Hilditch, 1949).

The fatty-acids most commonly occurring in fats have a long straight chain of carbon atoms with a single, terminal carboxyl group, the remaining valencies of the carbon atoms being satisfied with hydrogen, or closed by an ethylene linkage, for example:



Very few fatty-acids containing branched or ring systems occur in natural fats, and very few with an uneven number of carbon atoms, but a notable exception to this rule is iso-valeric acid ($C_5H_{10}O_2$), which is found in the depot fat of dolphins and porpoises. In the case of many fats originally thought to contain fatty-acids with an odd number of carbon atoms, it has subsequently been shown that

the acid in question was an equimolecular mixture of other two simpler acids; for example, daturic acid from datura oil, ($C_{17}H_{34}O_2$), has been shown to be an equimolecular mixture of palmitic acid, ($C_{16}H_{32}O_2$) and stearic acid, ($C_{18}H_{36}O_2$). Most of the fatty-acids occurring in natural fats belong to the series C_{12} , C_{14} , C_{16} , C_{18} , C_{20} and C_{22} , and the general formula for the saturated acids is $C_nH_{2n}O_2$ where n is an even number, and for the unsaturated acids $C_nH_{2n-2}O_2$, $C_nH_{2n-4}O_2$ etc., depending on the number of double bonds present in the molecule.

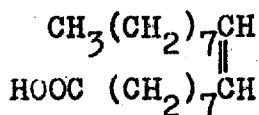
The Chief Fatty-acids of Natural Fats.

Of the saturated fatty-acids occurring in fats three are of particular importance, n-lauric acid, n-stearic acid and n-palmitic acid. n-Lauric acid has the formula $C_{12}H_{24}O_2$ and is widely distributed in plant fats such as coconut and palm oil, and occurs predominantly in sperm whale head oil. n-Stearic acid ($C_{18}H_{36}O_2$), is widely distributed and forms 1-5% of total fatty acids in most fats, and 10-30% of lards and tallow. n-Palmitic acid ($C_{16}H_{32}O_2$) occurs in fats from all sources, being the most abundant of the saturated fatty-acids.

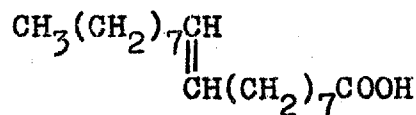
Of the unsaturated fatty-acids the most important member occurring in natural fats is

9:10 oleic acid ($C_{18}H_{34}O_2$). This acid exists

in two isomeric forms having the structural formula shown below:



Oleic acid (cis form)



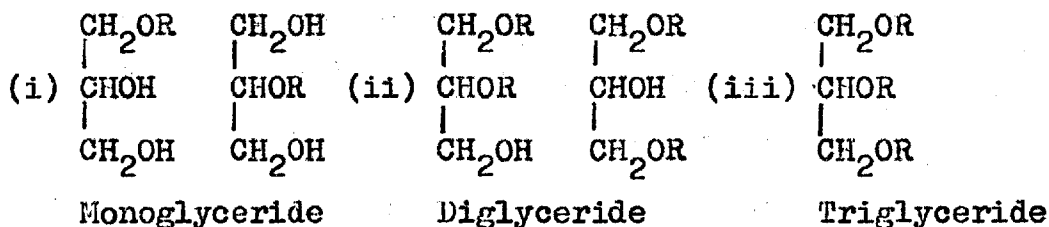
Oleic acid (trans form).
(Elaidic acid)

Saturated and unsaturated fatty-acids occur together in natural fats, the relative proportions determining the consistency of the fat. The higher the proportion of unsaturated acids present the softer is the fat.

The Alcohols of Natural Fats.

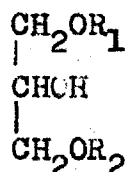
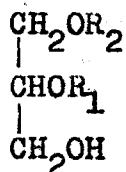
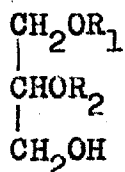
Fewer types of alcohols than of fatty-acids are combined in natural fats, the trihydric alcohol glycerol being by far the commonest. Glycerol occurs in practically all oils and fats, but not in waxes, and its decomposition product trimethylene glycol $\text{C}_3\text{H}_8\text{O}_2$, has been found in fat from many animal sources.

Glycerol has the formula $\text{CH}_2\text{OH}.\text{CHOH}.\text{CH}_2\text{OH}$, and its esters can take the following forms:

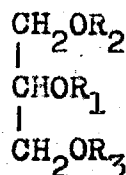
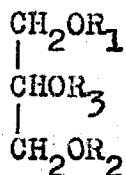
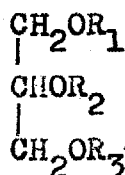


If each fatty-acid represented by R is different, for example, R_1 palmitic acid, R_2 stearic

acid and R_3 oleic acid, three isomeric diglycerides and three isomeric triglycerides could exist:



Diglycerides



Triglycerides

Monoglycerides have not been found to occur naturally in fats (Hilditch, 1947). Diglycerides are found in natural fats usually after some decomposition has set in. The triglycerides occur most commonly, usually as mixtures. Oleodistearin and oleodipalmitin occur in cocoa-butter fat (Fitzweiler, 1902), and stearodipalmitin occurs in duck and goose fat (Klimont, 1912). By using fractional crystallisation methods, Borner, (1913) and Amberger (1913 and 1918) proved that natural fats are composed of mixed triglycerides and showed the presence, among others, of palmitodistearin and dipalmitostearin in lard and butter fat respectively. Certain mono-diethylenic alcohols containing sixteen, eighteen and twenty carbon atoms in the molecule are found in some animal fats such as the oil of the sperm whale, while a small proportion of other alcohols of

as yet undetermined molecular structure, but clearly resembling the terpenes, occur to a varying degree in most natural fats. The proportion of the latter is usually less than one per cent of the total, except in wool fats and some liver oils where it is higher.

The alcohols found in waxes are mostly higher homologues of glycerol, usually saturated, and containing from twelve to thirty carbon atoms in the molecule.

A few sterols have been identified in natural fats. The phytosterols occur in plant fats, and the zoosterols, notably cholesterol, ($C_{27}H_{45}OH$), in animal fats.

The higher alcohols occurring most frequently in lipids are cetyl (n-hexadecyl), n-octadecyl and n-tetradecyl alcohols, in the saturated series, and oleyl (n- Δ^9 octadecenyl) in the unsaturated series. These correspond respectively in carbon content and chemical constitution with palmitic, stearic, myristic and oleic acids. In the waxes of plants and insects, the characteristic alcohols belong to the saturated series, and contain from twenty-six to thirty-six carbon atoms in the molecule, for example, ceryl and melissyl alcohols. It has been proved by synthesis that these are formed by the reduction of glycerides, simple esters and free fatty-acids, and also by the reduction of fatty-acid

esters (Bouveault and Blane, 1904). The saturated, higher aliphatic alcohols usually occur as a complex mixture, difficult to separate. Sometimes, exceptionally, one particular alcohol is present to the exclusion of all others: this is particularly true of leaf waxes. Some examples of these higher alcohols occurring in lipids are:

n-dodecanol $\text{CH}_3(\text{CH}_2)_{10}\text{CH}_2\text{OH}$, occurring in porpoise and whale oil.
n-hexadecanol (cetyl alcohol) $\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{OH}$, occurring in whale and fish oil.
Even-numbered alcohols $\text{C}_{26}\text{H}_{53}\text{OH}$ to $\text{C}_{36}\text{H}_{73}\text{OH}$ occur in wheat leaves, apple cuticle and cotton.
and $\text{C}_{24}\text{H}_{49}\text{OH}$ to $\text{C}_{34}\text{H}_{69}\text{OH}$ occur in beeswax.

The complex alcohol 15-keto-n-tetratriacontanol, $\text{CH}_3(\text{CH}_2)_{18}\text{CO}(\text{CH}_2)_{13}\text{CH}_2\text{OH}$ occurs in cochineal (Hilditch, 1947).

The Vitamins of Natural Fats.

Most natural fats contain varying quantities of the fat-soluble vitamins, Vitamin A which is an unsaturated alcohol of the carotene type, and Vitamin D which is a triethylene alcohol.

THE DISTRIBUTION OF FATS IN NATURE

Most natural fats are very complex mixtures, mostly of glycerol combined with fatty-acids. They have been described by Hilditch (1947) as "mixtures of mixed triglycerides". Usually five or more fatty-

acids are present in a fat, but in rare cases there may be only two. In cotton-seed and almond oil there are six and four respectively.

	<u>Cotton-seed oil</u>	<u>Almond oil</u>
Myristic acid	3.0%	1.0%
Palmitic acid	20.0%	5.0%
Stearic acid	1.0%	-
Oleic acid	30.0%	77.0%
Linoleic acid	45.0%	17.0%
Arachidic acid	1.0%	-

The same fatty-acids are widely distributed throughout natural fats; for example, the unsaturated hexadecenoic acid (palmitoleic acid, $\text{CH}_3(\text{CH}_2)_5\text{CH}:\text{CH}(\text{CH}_2)_7\text{COOH}$), occurs in lower aquatic and land animals, in yeasts such as Saccharomyces cerevesiae, in the bacterium Corynebacterium diphtheriae and in the cryptogam Lycopodium. In only a very few cases is a particular combination of fatty-acids unique, but such unique combinations do occur in certain plant seeds. This uniformity of distribution means that the differences between individual fats, and their specific properties are due to the variation in the proportion of fatty-acids present rather than to the type of component acids. It usually happens that, along with a particular fatty-acid its next higher and lower homologues are present also.

From various analyses it appears that the

most primitive organisms contain the most complex mixtures of fatty-acids, while the fats of higher organisms contain fewer fatty-acids, or at least fewer of the chief component ones.

In the plant kingdom most of the fats occur in the seeds and fruits, and the occurrence of specific fats depends mainly upon the climatic conditions which control the growth distribution of plants over the earth. It has been found that, in general, the fats from plants in tropical regions contain a lower proportion of unsaturated fatty-acids than do those of plants growing in temperate zones, probably as a result of the higher temperatures, and also a higher relative proportion of fatty-acids containing less than sixteen carbon atoms in the molecule. The waxes of plants are found extensively on the outer skin of leaves, stem, flowers and fruit. These waxes usually contain hydrocarbons of the paraffin series $C_n H_{2n-2}$ where n covers the range 29 to 59, along with alcohols of the phytosterol series, which may be free or combined with free fatty-acids and higher aliphatic alcohols such as ceryl alcohol ($C_{26}H_{53}OH$).

In the animal kingdom, fat varies slightly in composition according to its position in the animal body, the proportion of unsaturated fatty-acid components decreasing with increase of temperature,

from the body surface inwards, and also varies from one animal to another in the species. This latter variation occurs because the composition of fat depends to some extent on the food ingested, and on the life habits of the individual animal, but there is usually some modification of fat ingested, by breakdown and re-synthesis, to keep the fat characteristic of the species fairly constant. The milk fat of most mammals is similar, and differs from other animal and vegetable fats in containing a low percentage of butyric and the caproic acids, i.e. butyric ($C_4H_8O_2$), n-caproic ($C_6H_{12}O_2$), n-caprylic ($C_8H_{16}O_2$) and n-capric ($C_{10}H_{20}O_2$) acids. It has been found that the depot fats, or lards of pigs, cattle and sheep are similar, with small variations according to differences in diet in countries far apart, as in different continents.

The depot fats of all terrestrial animals show a gradual simplification of mixed fatty-acids, as compared with those of the aquatic animals. In the higher land animals the fatty-acids are predominantly saturated: palmitic acid ($C_{16}H_{32}O_2$) forms twenty-five to thirty per cent, and stearic acid ($C_{18}H_{36}O_2$) forms five per cent, of the total fatty-acids present. The chief unsaturated acid occurring in these sources is oleic acid ($C_{18}H_{34}O_2$).

In mammals fat is the chief form of reserve food material and it is contained in enlarged cells in the connective tissues, where the protoplasm of the cell has been altered to form a thin membrane surrounding each fat globule (Lovatt Evans, 1945).

The waxes of animals are present on the body surfaces as well as in the tissue cells. Hydrocarbons occur less frequently than in the plant waxes and the phytosterols are replaced by the cholesterol. The higher aliphatic alcohols present usually contain fewer carbon atoms than do those in plant waxes, generally having between sixteen and twenty carbon atoms in the molecule.

Fish oils are characterised by having a varying proportion of glycerides of highly unsaturated acids, containing twenty and twenty-two carbon atoms in the molecule, and by the glycerides of hexadeconoic acid ($C_{16}H_{30}O_2$). This latter acid occurs also in the whale and other marine animals.

In aquatic organisms the fats are characterised by a large proportion of unsaturated fatty-acids of the C_{16} , C_{18} , C_{20} , and C_{22} groups. Palmitic acid is the only saturated acid which forms a major component of these fats and it may account for eighteen to twenty per cent of fatty-acids in aquatic fats.

SOME PROPERTIES OF LIPASES

Lipases belong to the larger group of enzymes known as Hydrolases (Anderson, 1946). As a group lipases are specific, and hydrolyse only fats and esters, but within the group itself there is relatively little specificity, most lipases being able to hydrolyse almost any fat or ester. Notable exceptions to this rule are the mammalian lipases, such as liver lipase, which hydrolyses esters readily and fats slowly, and pancreatic lipase which has the reverse activity.

The lipases, like most hydrolases, are said to be extracellular, being secreted into the surrounding matrix, and their main function from the point of view of the organism producing them is to break down the molecules of complex food material into smaller ones which can be more readily utilised, and consequently relatively small energy changes occur during their activity.

Various theories have been put forward to explain the action of enzymes, but no completely satisfactory one has yet been found. In general, enzymes are regarded as organic catalysts, and their mode of action compared with that of inorganic catalysts. These latter act either through the formation of intermediate complexes which break down to give the end

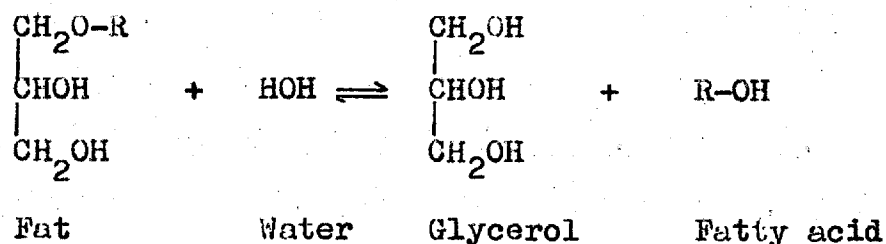
product and the free catalyst, or as carriers, thereby increasing the active concentration of one or more of the reactants. The theories most widely approved for enzymes involve the formation of complexes between the enzyme and its substrate, but there is much speculation as to the nature of these complexes. Michaelis and co-workers suggested that the enzyme and its substrate were in solution and that the reaction took place between their ions, but Bayliss (1914), thought that the substrate was adsorbed on the surface of the enzyme and that a chemical reaction took place on the adsorbed surface area. These theories both assume that enzymes are identifiable substances, and in opposition to them Quastel (1940), and his colleagues suggested that enzymes are not particulate, but rather a property of the cell surface. Using the theory put forward earlier by Wieland that hydrogen in a molecule can be readily activated, they suggested that electro-magnetic forces of attraction were set up between adjacent or closely situated molecules on the surface of a cell, and that as a result of these forces a region of concentrated energy is formed, which region is the enzyme itself. This "active centre" is then assumed to be able to adsorb the substrate molecules, and to activate them by distorting their electronic systems, and so render them unstable, and in this unstabilised state the molecules break down or alter to give the change

characteristic of the enzyme. According to this theory the specificity of enzymes depends upon the particular molecular groupings which are activated in the cell surface.

The physical and chemical stability of some bacterial lipases was investigated by Sohngen (1924), who showed that the lipases of Bacterium punctatum, Pseudomonas pyocyanea and Bacillus liquefaciens albus were more stable to heat than those of Bacterium lipolyticum (C. xerosis), Bacillus stutzeri, Pseudomonas fluorescens, Oidium lactis, Aspergillus niger, Penicillium glaucum and Cladosporium butyri. He also found two types of lipase, which differed in activity in the presence of acid, and which he designated the alpha and beta lipase.

THE THEORY OF BACTERIAL LIPOLYSIS

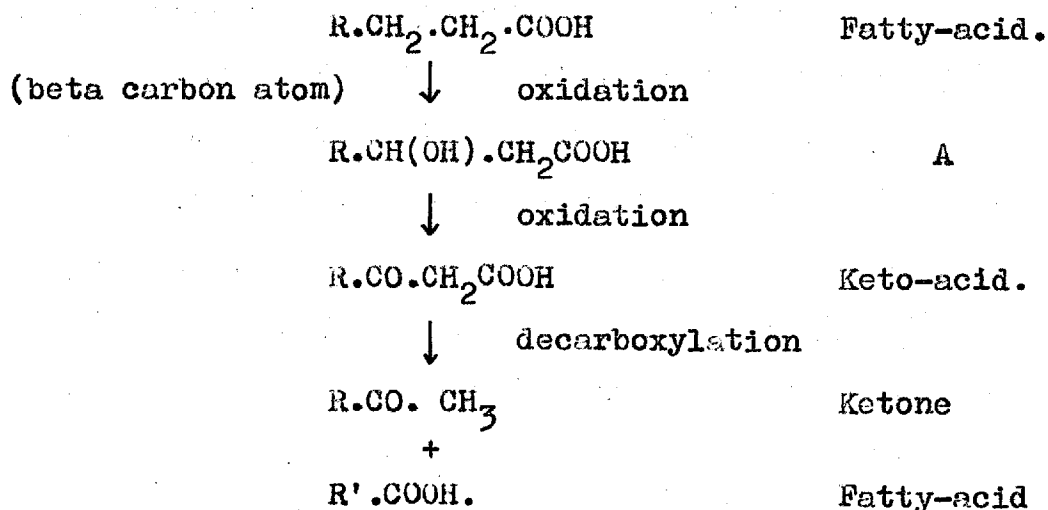
Early workers on bacterial lipolysis found that micro-organisms attack the fat molecules by hydrolysis, the fat being split, and water added on to give a free fatty-acid and glycerol. This reaction could be represented hypothetically as



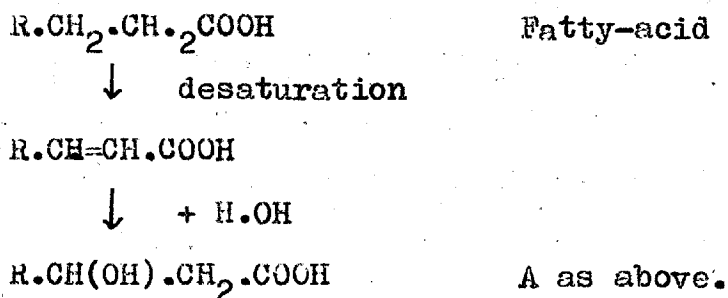
The glycerol thus formed is generally utilised as a source of carbohydrate and broken down further, usually to acetic and formic acids, sometimes with the liberation of carbon dioxide and hydrogen. The fatty-acid is sometimes oxidised to an alcohol (von Sommeruga, 1894; Rubner, 1900; Schreiber, 1902). Other workers (Rhan, 1905), considered that the fat molecules required to be oxidised first, to prepare them for microbial growth. Some workers, notably Walker (1906), found that moulds were the most active of micro-organisms in the utilisation of fats. Many years later Derx (1925) continued Walker's work and observed that moulds frequently utilised fats with the subsequent formation of ketones. This could occur if fats were primarily oxidised in the beta position in the molecule, and by

the splitting of the beta-keto fatty-acids thus formed into methyl ketones and carbon dioxide. He also found that up to and including myristic acid in the homologous series, the oxidation proceeded no further than the fatty-acid, because the fatty-acids formed were adsorbed on to the mould mycelium, thereby hindering further metabolism. Stokoe (1928) confirmed Derr's work on the cleavage of the fat molecule at the beta carbon atom, and showed that, when certain moulds, for example Penicillium glaucum, grow on coconut oil they produce methyl, amyl, methyl-heptyl and methyl-nonyl ketones. Later Taufel, Thaler and Loweneck (1936) found that, within certain limits of acidity, Penicillium glaucum and Aspergillus niger formed methyl ketones from some fatty-acids but not from palmitic, stearic and oleic acids. The results of these observers also showed that the mechanism of breakdown of fat molecules by micro-organisms is similar to that of man and the higher animals, in whom cleavage of the fat molecules takes place at the beta carbon atom to give beta-keto acids. These break down further to give fatty-acids with two carbon atoms fewer than the parent keto-acid, and an organic acid which is generally acetic acid (CH_3COOH). From these fatty-acids ketones are formed, and along with them, esters of ethyl and secondary alcohols are usually produced. In the dog, for example, it has been shown by Dakin (1908) that the breakdown of

fatty-acid in the intestine probably takes place in the following stages:

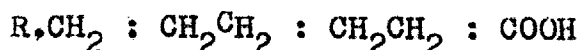


Some workers believe, however, that desaturation of the fatty-acid takes place first of all with the formation of a double bond, before further breakdown to the ketones and fatty-acids (Leathes and Weddell, 1909). Their mechanism would therefore be as follows:



This mechanism seems probable, in theory at least, but experimentally none of the intermediate products has been detected in vivo, only the original

acids and the end-products, carbon dioxide and water, being found. Because of this it was concluded by Jenett and Quastel (1935) that the breakdown of fatty-acids was effected by the oxidation of alternate carbon atoms along the chain, for example:



Other workers, Mackay, Arne, Carne and Barnum (1941) considered that the terminal carbon atoms were split off first, to form acetic acid, the molecules of which then gave acetoacetic acid by aldol condensation.

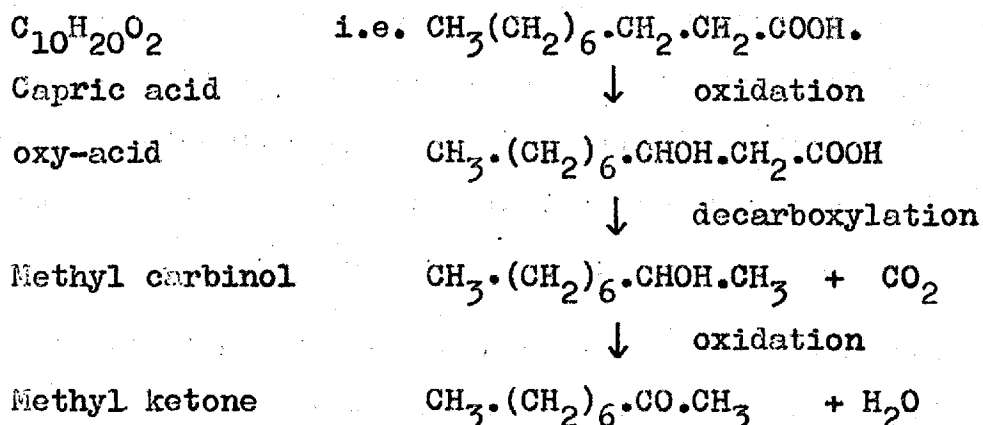
Others again, Weinhouse, Medes and Floyd (1945) investigated this mechanism by using the heavy isotope of carbon C_{13} . They concluded that the chain of the fat molecule is broken up in two ways, primarily into smaller units of chains, of which some recondense, and secondarily, and to a lesser extent, broken by oxidation of the chain at the beta carbon atom.

The above investigations have been repeated by other workers, and much more work done, on the mechanism of fat decomposition, but nevertheless much uncertainty remains. Silliker and Rittenberg (1951) summarise the current opinions in their papers on the oxidation of fatty-acids by bacteria. They state that fatty-acids may be toxic, stimulating or major energy-yielding substrates for bacteria, and that there are three possible operative mechanisms of breakdown, which they designate by the terms beta, multiple alternate and

omega oxidations, depending upon the position in which the carbon chain of the fatty-acid molecule is broken by the bacterium. They suggest that technical difficulties in detecting very low concentrations of suspected intermediates or the toxicity or low solubility of these products, may be responsible for failures to detect them in culture filtrates or in mixtures from analyses. It has, however, been established that where the fatty-acids liberated are toxic, and they are adsorbed by the micro-organisms, as they frequently are by the moulds and fungi, an abnormal form of fat decomposition is induced so that the organisms can obtain energy from the deposited fat, and the keto-acids formed are decomposed to methyl ketones and carbon dioxide.

The formation of ketones by moulds was further investigated by Davies (1928, 1929, 1930, 1932). In his first paper (1928) Davies divided lipolytic moulds into two classes, the "Dry" moulds and the "Oily" moulds. Moulds belonging to the "Dry" group modify their metabolism as their mycelium becomes coated with an adsorbed layer of fat or fatty-acids. They utilise the fatty-acids by oxidising them at the beta-carbon atom to give another acid lower in the homologous series, having two carbon atoms fewer in the molecule. This decomposition process can then go further with the production of a methyl ketone and carbon dioxide.

For example, capric acid can be broken down as follows:



In the case of capric acid the ketone formed is methyl heptyl ketone. Similarly, lauric acid, $C_{12}H_{24}O_2$ finally gives methyl nonyl ketone $CH_3 \cdot (CH_2)_8 \cdot CO \cdot CH_3$. Moulds belonging to the "Dry" group are strains of *Penicillium*, *Aspergillus* and *Monilia* (*Candida*), and they can utilise fatty-acids containing up to ten carbon atoms in the molecule. The "Dry" moulds have no apparent lipase, and when growing on a fatty substrate behave as they would on an unfavourable medium.

Moulds belonging to the "Oily" group do produce a detectable lipase, and ferment the fat with the liberation of oleic acid, which gives the matrix an oily appearance. Examples are strains of *Oidium* and *Oospora*. This group grows well and continues to utilise the fat to an advanced stage of its growth.

In a later paper Davies (1932) suggested two explanations for the action of micro-organisms on fatty substrates;

(i) that it is the result of competitive demand for

oxygen for their respiration;

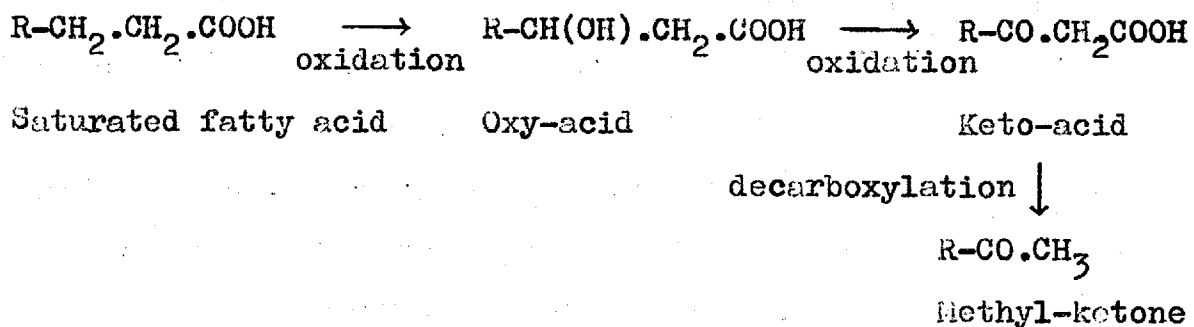
(ii) that it is due to the action of acids produced by the organisms during their metabolism.

The first mechanism is important where the preservation of the fatty substrate is concerned, because activated or ionised oxygen must be available before the fat can break down. If there is insufficient activated oxygen left over after the requirements of the micro-organisms have been satisfied, no breakdown of the fat occurs, and therefore, the fatty substrates have been protected from microbial action by the absence of oxygen. If the second mechanism is the operative one, however, relatively small numbers of micro-organisms can produce sufficient acid by fermentation to promote the breakdown of the fat. The lactic acid produced in milk is an example of this mechanism in a very active form.

In the following year Jensen and Grettie (1933) described two classes of microbial enzymes for fatty substrates, oxidases and lipases, and they showed that bacteria which have both types can produce free fatty-acids and oxidation products. Carrying this investigation further, Horowitz-Wlassowa and Livschitz (1935) found that the majority of micro-organisms hydrolyse the fats first then oxidise the products, but that a few species, for example, Bacterium lactis aerogenes, are able to oxidise them directly.

The oxygen taken up by unsaturated fatty-acids to form peroxides was estimated quantitatively by the liberation of iodine from potassium or barium iodide, using potassium thiocyanate and a starch indicator, Wheeler (1932). It has also been estimated by photo-electric colourimetry, by the oxidation of ferrous iron salts, and the subsequent formation of a coloured ferric thiocyanate complex, Sumner (1943).

Hilditch (1949), considering these and other theories, states that moulds such as *Penicillium* and *Aspergillus* which produce lipoclastic enzymes, that is, lipases, split the fats by hydrolysis with the liberation of free fatty-acids. Those organisms which produce enzymes of the peroxidase type, oxidise saturated as well as unsaturated fatty-acids, breaking the molecule at the beta-carbon atom from the carboxyl group, with the formation of ketonic acids, and ultimately of methyl ketones; for example:



This type of oxidation reaction occurs in conditions of moderate temperature and slight humidity, and most readily on fatty-acids containing between eight and twelve carbon atoms in the molecule. While it is

important among the actions of micro-organisms on fats it is less common than the direct oxidation of unsaturated fatty-acids in presence of atmospheric oxygen, with the formation of intermediate unstable peroxides. The exact mechanisms by which these peroxides subsequently break down to give the final end-products have not yet been established. Sufficient experimental evidence has, however, been obtained from fat chemistry to show that micro-organisms do act upon fatty substrates by means of their hydrolytic and oxidative enzymes. Either type of enzyme may act alone or predominantly, or both types may occur together, acting simultaneously but independently.

These processes have been rechecked and summarised by Muckherjee (1951). They are stated by him to occur in the following order: hydrolysis of glycerides to free fatty-acids and glycerol by lipases; rapid oxidation of the individual components - the lower saturated fatty-acids - by acid oxidases; oxidation of unsaturated fatty-acids by lipoxidases; desaturation of saturated acids by dehydrogenases followed by oxidation by oxidative enzymes. Secondary decomposition of the peroxides formed takes place by lipoxidase action, and by auto-oxidation by peroxidases. All these reactions proceed simultaneously, making the resulting mixture a very complex one.

Bacterial Lipolysis in vitro

According to Collins and Hammer (1934), the majority of micro-organisms which can hydrolyse one natural fat or oil, can hydrolyse most others. When they tested ninety-two strains of different micro-organisms on media containing ten different natural fats, such as olive and linseed oil, between 72% and 86% of the organisms were able to utilise all of the fats. When simple triglycerides were used instead of fats, however, there was a much greater variation in the proportion of micro-organisms which could attack the different triglycerides. Collins and Hammer tested one hundred and nineteen strains of micro-organisms against thirteen triglycerides and found that, while most strains could hydrolyse all or most of the thirteen triglycerides to some extent, 98% of them could hydrolyse tripropionin, while only 2% hydrolysed tripalmitin. This showed that some triglycerides are more readily hydrolysed than others.

Bacterial lipolysis can be demonstrated in vitro in several ways, of which titration of increased acidity in fluid medium, and the appearance of a zone of clearing or colouration round the colony on solid medium, are the commonest and probably the most reliable. Other methods available include the use of fat emulsion agar with an

indicator for acidity such as litmus or bromophenol blue, as utilised by many workers such as von Sommeruga (1894), Buchanan (1921) and Collins and Hammer (1934). The use of indicators such as Nile Blue sulphate, specific for fatty-acids, was favoured by Smith (1908) and Turner (1929), while the formation of blue-green soaps with copper sulphate is described by Berry (1933). Eyre (1930) added a suspension of erythrocytes to sugar-free fat-emulsion broth filtrates, and claimed that haemolysis was an indication of the presence of lipase in the original culture, while Michaelis and Nakahara (1923) used the stalagometric method of Rona and Michaelis (1911). Published literature also contains references to the use of the diphenylamine and nitric acid test for free fatty-acids, and to the formation of coloured soaps between fatty-acids and metal radicals, Jensen and Grettie (1937); to the electrotitrimetric method of Rona and Ammon (1927); to the gasometric method using the Warburg apparatus, Rona and Lasnitski (1924), and to the nephelometric method of Rona and Kleinmann (1926). Dialometric methods, measuring volume changes during hydrolysis have also been tried, but not found very satisfactory. (Rona and Ammon, 1932). On solid medium whether the indicator of choice be a zone of clearing or of colour development, depends upon the fat incorporated. Some fats such as tricaproin and tricaprylin hydrolyse to give products which do not stain well with fat dyes, and

hence the hydrolysis is more easily observed by the disappearance of the fat globules and the formation of a clear zone. With others, however, for example tricaprin, the reverse is true. The formation of a clear zone is preferable to a coloured one also in cases where the fatty-acids liberated by hydrolysis are comparatively soluble in the matrix and diffuse rapidly through it, because the colour produced with dyes is less intense owing to the diffusion.

The method of incorporating the lipid in the matrix is of fundamental importance, as there is an optimum rather than a maximum surface area of the fat globule which should be presented to the micro-organisms. A few triglycerides, for example tributyrin, are soluble in water, but the majority, along with the natural fats, are not, and must be introduced into the medium as suspensions. The ease with which these suspensions form decreases with increase in the molecular weight of the lipid. Various agents can be used as suspenders, but when the common organic solvents, such as ether, benzene, petroleum or carbon tetrachloride are considered, their possible inhibitory or toxic effects on bacteria must be borne in mind. One of the safest and most efficient emulsifying agents is 0.5% aqueous agar, which gives a uniform and stable suspension; (Collins and Hammer, 1934).

The reaction of the medium in which lipases have been found to act may vary from pH.5.0 to PH 8.0

but the optimum is on the alkaline side of neutrality.

The temperature range over which lipolysis occurs seems to be wide. Collins and Hammer (1934), carried out most incubations at 21° C., but according to Chalmers (1945) lipolysis occurs most commonly around 10°C. Some workers studying lipolysis in products in cold storage have found it to occur below freezing-point. These observations may in part explain the rarity with which lipolysis is encountered among parasites in species which have a higher optimum growth temperature of 37°C.

When lipolysis is displayed by a micro-organism all the fat globules surrounding the colony may be hydrolysed to the same extent, and stain uniformly on a colour reaction, when the lipolysis is said to be uniform. If the surrounding globules are not all attacked similarly by the lipase, the staining is unequal, and the lipolysis is said to be non-uniform. In both these groups there is considerable variation in the extent of diffusion of the lipase around the colony, some weakly active lipolytic organisms confining their activity to the matrix directly below the colony, while strongly active organisms produce a lipase which spreads around the colony to give a zone several millimetres in width.

The technical aspects of these latter considerations will be described in more detail in the sections dealing with experimental work.

SURVEY OF PUBLISHED WORK ON BACTERIAL LIPOLYSIS

Following the work of Escherisch (1886) and Nencki (1886), already quoted, Sommaruga (1894) grew various strains of bacteria on solid medium in which was incorporated 2% olive oil. He claimed that when the fat was hydrolysed by bacteria the fat globules disappeared from the area of the plate surrounding the colonies. His work was followed up by Eijkman (1901), who poured inoculated, nutrient agar over a thin layer of tallow in a Petri dish and detected the formation of lipase by the bacteria by the formation of a clear zone in the tallow layer immediately below the colonies producing the lipase. de Kruyff (1906) gave the name of "Lipobacteria" to those organisms producing a fat-splitting enzyme and in 1908 investigated their oxidative action on fats.

Sahr, Rhan and Farrand (1908) were among the first to publish work in which dyes were used to detect lipolysis. They used a suspension of butter fat in sugar-free broth, to which they added agar and litmus. The action of bacterial lipase was shown by a change in the colour of the litmus indicator.

In the same year Smith (1908) published some work on the reaction between neutral fats and free fatty-acids and the aniline dyes, notably Nile Blue sulphate. This dye was shown to be pink when in contact

with neutral fat, and blue with free fatty-acids. His work was followed up later by Boeminghaus (1920), who studied the reaction between Nile Blue sulphate and palmitin, stearin and olein and their derivatives. He found that olein and its acids and esters gave much more vivid colour reactions than did palmitin or stearin.

In the interval between the work of Smith and Boeminghaus, Wells and Corper (1912) examined strains of *Staphylococcus*, *Pseudomonas*, *Escherischia*, *Shigella* and *Mycobacterium*, killed by toluene and suspended in medium containing ethyl butyrate, triacetin or olive oil. They found these organisms to possess lipases in the above order of decreasing activity. Only the first two genera showed lipolytic activity when growing on plates of fat medium, but all genera showed it when the killed cultures were suspended in contact with fats and esters.

Kendall, Day and Walker (1914) and Kendall, Walker and Day (1914) studied the lipolytic action of bacteria on milk fat, by cultural diffusion methods, and found two active species, *Proteus vulgaris* and *Bacillus mesentericus*. They also investigated the production of lipases by various strains of *Mycobacterium*, using cultural diffusion and chemical extraction methods, and described two types. One of these was retained in the bacterial cell, as an endo-enzyme, and the other was secreted into the medium as an exo-enzyme. These workers observed that the period of maximum production

of the lipase coincided with that of maximum vegetative growth of the cells, and was proportional to the luxuriance of the growth. Kendall and Simonds (1914), using the same chemical extraction method, observed the production of lipase by some strains of *Salmonellae*. Avery and Cullen (1920) investigated the enzymes of the *Pneumococcus*, by chemical extraction methods, including the action of its lipase on tributyrin. They estimated the free fatty-acids liberated, by titration with 0.1 N sodium hydroxide. They found that young cultures contained no free lipase and that lipase was liberated only by autolysis. This lipase was thermolabile and its production was unrelated to the virulence of the organism as its activity persisted after virulence had been lost.

Stevens and West (1922) published results of work on three enzymes of haemolytic streptococci, the peptase, the invertase and the lipase. They extracted the enzymes by grinding the organisms in one-fifteenth molar phosphate buffer and investigated their action in vitro. They found the lipase to have a maximum activity in solutions of pH 7.9 and to be thermolabile.

The lipases of moulds were investigated by Schenker (1921) using *Aspergillus niger*, and by Derx (1924; 1925), while Michaelis and Nakahara (1923) made a wide search for a lipase in different species of

bacteria and fungi. They used butter-fat and tributyrin as substrates, and measured the lipolytic activity of the chemically extracted enzymes stalagometrically. They found that the acidity range over which the lipases were active varied from pH 7.2 to pH 9.0. The optimum for yeast was pH 5.8 to pH 6.2. Only some of the lipases were thermolabile. These workers claimed to have demonstrated lipases in strains of *Pseudomonas*, *Streptothrix*, *Oidium*, *Penicillium* and *Mucor*, all of which were isolated from soil sources, and also in strains of *Bacterium*, *Pseudomonas*, *Streptococcus* and *Micrococcus* which they isolated from faeces of breast-fed infants, and in strains of *Mycobacterium* and of the pyogenic cocci. They failed to demonstrate a lipase in strains of *Bacillus subtilis*, or of the *Corynebacteria*, *Pneumococci*, *Salmonellae* or *Shigellae* or in *Bacterium* from adults.

Orcutt and Howe (1922) sought for a relationship between the haemolytic activity of staphylococci and the presence of a lipase, using a medium containing cream and blood. They concluded that some strains were haemolytic only when their lipase was active, the haemolysis being due to the lytic action of the free fatty-acids liberated,

Van der Walle (1927) confirmed earlier workers' claims by finding that a lipase was present in cultures of staphylococci and of *Pseudomonas pyocyanea*, and in some strains of coliform and dysentery bacilli. The lipases were extracted chemically and their lipolytic

activity against tributyrin measured stalogometrically.

Further work was then published on the dye Nile Blue sulphate. Kaufmann and Libman (1926) found that it would stain saturated and unsaturated fats, while Kettie (1931) separated it into its two component parts, Nile Pink and Nile Blue, of which only the latter is soluble in water.

Workers were at this time tending to seek for a solid medium which would demonstrate lipolysis satisfactorily. Buchanan (1921) continued to use the disappearance of the fat globules around the bacterial colonies as the criterion of their lipolytic activity, but Waksman and Davison (1926) favoured the use of indicators to detect the free fatty-acids liberated when the bacteria hydrolysed the fat.

Turner (1928) used a solid nutrient agar medium containing an emulsion of various triglycerides and natural oils, and an aqueous solution of Nile Blue sulphate, the properties of which he investigated. Later (1929), in a comparison of the various methods available, he concluded that Nile Blue sulphate medium gave the greatest sharpness of differentiation, but that, if a medium without the presence of a dye was required, an emulsion of short-chained triglycerides, or of an oil such as cottonseed oil, used in conjunction with bile salts, gave the ^{best} results. His summary

is reproduced in Table I.

Table I.

Group No.	Name of Medium	Fat	Dye or Indicator	Quality on which differ'n depends	Sharpness of differ'n	Days necessary for differ'n	Inhibition of bacterial growth	False Positive reaction
1.	Sijkman	Beef tallow	None	Opacity or translucence	Poor	4	None	None
2.	Buchanan	Cotton seed oil	None	Opacity or translucence	Poor	6	None	None
3.	Sudan IV	Emulsion of stained cotton seed oil	/	Opacity or translucence	Poor	2-4	None	None
4.	Hydrogen ion	Cotton seed oil	Litmus: Bromothymol blue	Colour change dependent on pH change	None	-	None	Due to carbohydrate
5.	Mile blue sulphate	Cotton seed oil emulsion	Mile Blue Sulphate	Blue zone due to staining of fatty acids.	Excellent.	12-18 hours	Certain organisms.	Fading zones cause some trouble

Turner's work was later modified by Hussong (1931), who used an alcoholic solution of Nile Blue sulphate, and emulsified the fat to be tested in one-half per cent agar.

Neill and Fleming (1927) studied the maltase and lipase of Clostridium botulinum by using the whole supernatant fluid of eighteen-hour cultures and also after extracting the enzymes by alternate freezing and thawing of the bacilli. They incubated the enzyme preparations in contact with a suspension of 2.0% tributyrin in 0.1 molar phosphate solution at pH 7.8, and after forty-eight hours estimated the acid liberated by the lipase, by titration. They found that the lipase was extracellular, in contrast to the maltase, and that it did not deteriorate on exposure to air.

Waksman (1929), in an investigation of soil saprophytes found that fungi were more active as fat splitters than bacteria, and that ketones were frequently formed among their reduction products. Among the lipolytic bacteria he isolated were strains of Staphylococcus aureus, Chromobacterium prodigiosum, Pseudomonas pyocyanea and fluorescens and "B. lipolyticum."

Harden (1930) contributed work on the lipolytic activity of intestinal commensals and corroborated Nencki's finding that they are not active in fat metabolism in the intestine. He verified earlier

observations that in presence of oxygen, the bacteria hydrolyse fats to glycerol and fatty-acids, with a further decomposition of the glycerol and oxidation

ERR.: Harden of the fatty-acids. He found that yeasts and moulds reviewed this work, but did not participate in it.

Sohnngen (1930) found that many bacteria can utilise fats as their sole source of carbohydrate, provided they are given the necessary source of nitrogen, for example, ammonium chloride.

ERR.: Eyre referred to this technique, but did not develop it.

Eyre (1930) continued work relating haemolysis to lipolysis by adding red blood cells to filtrates of sugar-free broth cultures of various organisms, and claimed that haemolysis of the blood cells indicated the presence of a lipase.

A new alternative method for detecting bacterial lipase was devised by Berry (1933), using Carnot's and Maubin's test (1918) for free fatty-acids. He incorporated the fat in a medium and incubated the inoculated plates until good growth was obtained, then poured a saturated solution of copper sulphate over the surface. Where the fat had been hydrolysed, an insoluble blue soap formed between the free fatty-acids and the copper sulphate, around the colonies.

Collins and Hammer (1934) published two papers in which they repeated Turner's work and confirmed his

conclusions. They found that many common bacteria, genera unstated, were unable to attack fats, but of those which could, all had much the same action on all neutral and hydrogenated fats tested. The blue colour given by Nile Blue sulphate was again shown to be due to the formation of soaps. The hydrolysis of simple triglycerides by bacteria was not appreciably affected by the way in which the fats were dispersed, nor by the reaction of the medium within the limits of acidity pH 5.3 to pH 7.8, but it was shown to be progressively more difficult for bacteria to hydrolyse fats with increase in their molecular weight. In their second paper Collins and Hammer (1934), using saprophytes from air and water and bacteria from dairy products, showed that there might be considerable variation in the type of lipolysis produced.

Anderson (1934) prepared a medium, tributyrin agar (0.1%) which he claimed was highly selective for lipolytic bacteria. He added various indicators, bromo-thymol blue, bromo-cresol purple and phenol red to detect acid production. Turner (1929) had found that such a medium as this did not support growth well.

Horowitz-Wlassowa and Livschitz (1935) used 10.0% milk agar and soya bean oil in an inorganic basal medium. They made very careful investigations of the chemical properties of the fats before using them, and looked for relationships between these properties and

the readiness with which bacteria could attack them. They found that if an organism produced both an oxidase and a lipase, the lipase acted first. An exception was Bacterium lactis aerogenes, which oxidised the fats directly. These workers detected lipolysis by the formation of zones of clearing round the colonies.

Stark and Schreib (1936) used a modification of Anderson's tributyrin agar, and tested four hundred and eighty-six strains of organisms, Gram-positive and Gram-negative cocci, Gram-negative bacilli and Sarcinae, responsible for the splitting of fat and digestion of casein in butter. Of these, 96.0% were able to hydrolyse tributyrin, a zone of clearing round the colony being taken as the indication of lipolysis.

Trussell and Weed (1937) examined the effect of some staphylococci on pure triglycerides. Their aim was to find some correlation between the lipolytic activity of a staphylococcus and its virulence, but no such correlation was found. They used a fluid medium at pH 7.4 to 7.6, and tested forty strains of staphylococci and two strains of micrococci against eight pure triglycerides. They used bromo-thymol blue as indicator, with normal sodium hydroxide, in a titration method. They found that practically all the organisms tested hydrolysed the fat to the same extent, and that the degree of lipolysis seemed proportional to the profusion of the growth.

Jensen and Grettie (1937) in a review survey the work done and the knowledge gained up to that time concerning the lipolytic activity of micro-organisms. They repeated an earlier claim (Jensen and Grettie, 1933) that the two classes of enzymes mainly responsible for bacterial action on fats are lipases and oxidases and they substantiated the work of Horowitz-Wlassowa and Livschitz (1935). They considered Turner's methods to be the most practicable and satisfactory, even although the high content of active oxygen present in the molecules of the cottonseed oil he used so frequently is inhibitory to many bacteria. They worked out their own medium which closely resembled Turner's, but they used coconut oil with gum tragacanth as suspender. They incorporated Nile Blue sulphate as indicator, and frequently counted the colonies on their plates, stating their results as the number of lipobacteria per gram of fat. They also used sodium oleate or taurocholate (0.1%) in preparing the fat emulsion.

Birch-Hirschfeld (1937) continued the attempt previously made to correlate lipolysis and haemolysis in staphylococci. She tested thirteen strains for lipase production on tributyrin, and for haemolysin production against sheep red blood cells. She found that the ratio of the amount of lipase to the amount of haemolysin produced was fairly constant, and she deduced from this that these two enzymes had a common

origin.

Christie and Graydon (1941) investigated the alpha lysin of staphylococci which can lyse both sheep and rabbit red blood cells and the beta lysin which gives hot-cold lysis of sheep red blood cells. The organisms were grown on cream blood agar, and haemolysis used as the indicator of lipolysis. In a second series of experiments butter fat (0.1%) in nutrient agar with neutral red (0.005%) as indicator, was used. The particles of hydrolysed fat turned red after two days of incubation at 37° C. They found that lipase production was independent of haemolysin production, which is in disagreement with Birch-Hirschfeld (1937), but using coagulase production as the criterion of pathogenicity, they found that lipase production is unrelated to pathogenicity, which is in agreement with the views of Trussell and Weed (1935).

Knaysi (1941) carried out a survey of the use of basic dyes for the demonstration of bacterial lipase, and found that, provided the concentration of the dye be kept below the toxic level, a number of basic dyes in which the free base is different in colour from the salts, are equally suitable. He suggested Nile Blue Sulphate, Neutral Red, Methylene Blue and Malachite Green. He emphasised that the medium must be neutral or slightly alkaline, and completely free from fermentable carbohydrates.

Ciaccio (1944) examined various strains of the gonococcus using a chemical method, but found "no esterase activity" in them.

Only a few other recent publications have been found; one paper by Govan (1944) on the chemistry of the dye Nile Blue sulphate stated that some of his results were confusing, as the fat globules, although pink when first stained, became bluish-purple on prolonged contact with the dye. Govan used the dye in a concentration of 0.2 per cent mainly for staining histological preparations.

Jezeski, Halvorson and May (1950) published a paper on the uptake of oxygen by bacteria in presence of lipid substrates.

Mukherjee (1951; 1952) published papers on fat degradation by micro-organisms. He gave a brief review (1951) of work already published and stressed the contradictory nature of results found by different workers, and the irregular distribution of lipolysis occurring "even with one and the same species within wide limits". He used the method of Turner (1929) for demonstrating lipolytic activity of the growing organism, mainly species of *Penicillium* and *Aspergillus*, and investigated the chemical nature of the intermediate products formed when these moulds grew on butter-fat and on the pure synthetic glycerides monobutyrim, tributyrin, tristearin and triolein.

Ramakrishnan and Banerjee (1951; 1952) studied the action of mould lipase on groundnut oil, while Cutchins, Doetsch and Felczar (1952) investigated the influence of the composition of the medium on the production of lipase by strains of *Pseudomonas* and of *Alcaligenes viscosus*. They treated a fluid culture with formaldehyde, and incubated it for twenty-four hours in contact with fat or a triglyceride, and titrated the acid liberated with alcoholic potassium hydroxide.

An analysis of the published work on bacterial lipolysis seems to indicate that the apparent discrepancies among the results of different workers may largely be due to the fact that bacterial lipase can occur either as an exo or an endo-enzyme, both classes of enzyme occurring together in some strains, and that this fact has not been fully recognised by all workers, using different methods to demonstrate lipolysis. It would appear that the exo-enzyme only is demonstrated by methods involving the development of a zone of clearing or colouration round the colonies of actively growing organisms, or by titration of acidity in growing cultures in fat-containing medium.

The differentiation of lipases into two classes is analogous to the well recognised classification of bacterial toxins as exo or endo-toxins, although as with these, the distinction is not absolute. Owing to the

methods used in this work, only the lipases of the exo-type have been studied.

The text-books consulted for the chemistry of fats and enzymes were Bloor (1943), Hilditch (1947), Lovatt Evans (1945), Sumner and Myrback (1950) and the monograph by Jamieson (1943); for bacteriological chemistry Anderson (1946) and Stephenson (1949); for systematic bacteriology Topley and Wilson (1946) and Salle (1943), and for some classification Bergey (1948).

RECORD OF EXPERIMENTAL WORK DONE

Part I.

1) REPRODUCTION OF TURNER'S WORK

Turner's Medium.

Formula (Turner, 1929).

Basal medium:

Bacto agar (Difco)	30.0 grams.
Disodium phosphate	5.0 "
Beef infusion broth	1,000 millilitres.

The final reaction was adjusted to pH-7.5, and the medium tubed in volumes of 5.5 millilitres and autoclaved.

Fat emulsion.

Cottonseed oil (Wesson oil)	100.0 millilitres.
India gum	3.0 grams.

These were thoroughly mixed with a motor-driven egg-beater, 200.0 ml. of hot distilled water added at once and the suspension mixed at high speed for five minutes. The resulting emulsion was tubed in volumes of 5.0 ml. and sterilised by autoclaving for fifteen minutes at fifteen pounds pressure, (approximately 120.0° C.).

This method, according to Turner, gave a stable emulsion with most of the fat globules less

than ten microns in diameter. This emulsion was added to the melted agar just before the plates were poured, in the proportion:

emulsion	0.75 ml.
basal medium	<u>5.5</u> "
	6.25 "

Dye solution.

Nile Blue sulphate	0.1 gm.
Distilled water	100.0 ml.

This solution did not require sterilisation or refrigeration and was stored in a stoppered, dark glass bottle at room temperature. It was added to the medium just before pouring the plates, in the proportion:

Basal medium and emulsion	6.25 ml.
dye solution	<u>0.75</u> ml.
	<u>7.0</u> ml.

Turner appears to have inoculated the medium before pouring the plates and so obtained colonies throughout. He usually prepared series in duplicate, incubating one aerobically and the other anaerobically, at 37°C. for forty-eight hours, and then re-incubating for a further two to three days. The anaerobic cultures had to be left at room-temperature for a few minutes for oxidation of the leuco-base of the Nile Blue sulphate, with

consequent development of the blue colour. His test organism was one isolated from the gastric contents of a patient suffering from pellagra. It was designated J.M.F. 6, but not identified. His negative control was a strain of Bacterium coli found by prolonged testing to be non-lipolytic.

Strains showing positive results produced a blue-coloured colony, usually surrounded by a clear zone, coloured blue. In some cases the colour was green or greenish-blue. Those strains which showed a very bright blue colony produced a wide zone of clearing in the medium round the colony, while those which were paler in colour produced correspondingly narrower zones. Feebly lipolytic colonies tended to show a zone of precipitation rather than of clearing, presumably due to incomplete hydrolysis, only some of the fat molecules around the colonies being attacked by the lipase.

Turner found that the time taken for the development of colour and zone of clearing by the colony and in the surrounding medium varied with the length of the carbon chain in the fat molecule, being shortest with the shortest chains. An average time was forty-eight hours. This time lag could be shortened, even halved, by the addition of sodium taurocholate (5.0%), and with its use the zones became crystal-clear, instead of being merely translucent.

He offered three possible explanations for this:

- 1) Bile salt may increase the diffusion of the neutral fat and provide smaller masses of fat and larger surface area.

- 2) Bile salt may accelerate the diffusion of free fatty-acids and soaps formed and so promote their removal from the vicinity once formed.

- 3) Bile salt may increase the growth of some organisms. It has been suggested by later workers (Sumner and Myrbeck, 1950) that bile salts function as activators for lipases.

Turner sometimes found that the concentration of dye used was inhibitory, two strains of Clostridium welchii and two of Staphylococcus aureus being inhibited by a dilution of 1 in 8,000 of Nile Blue sulphate in solid medium. He overcame this by flooding the surface of the plate with dye after incubation.

Using his organism J.M.F. 6, Turner corroborated Eijkman's view that organisms which produce much alkali in their metabolism and can therefore form soaps, do not split fats by means of this alkali. Alkali producers which can split fat do so by producing a lipase.

Turner watched the action of bacterium J.M.F. 6 on fat globules incorporated in a layer of agar medium containing Nile Blue sulphate and held between a microscope slide and cover slip, and he made photomicrographs of his observations. He found that lipase diffused from the bacterium and hydrolysed the fat with the formation

of unsaturated free fatty-acids, which were stained blue. Some of these fatty-acids, notably oleic and linoleic, formed soaps, and these diffused into the agar. The fat globule shrank and became crescent-shaped, and in some cases fine crystals protruded from the shrunken globule. These crystals were needle or prism shaped, and varied in number. Sometimes they were seen well removed from all fat globules. When examined with polarised light most were doubly refractile. They were composed of solid fatty-acids, palmitic and stearic, or their derivatives, and were formed from forty-eight hours of incubation onwards.

When investigating the coli-anaerogenes group, using Nile Blue sulphate, Turner recorded the appearance of brilliant blue or purple zones, which faded in one to two hours. He found no gross disintegration of the fat globules in agar medium, and no increase in the titratable acidity when killed cultures were allowed to act on tributyrin. He offered no explanation for these temporary zones.

Attempts to repeat Turner's work gave only moderately satisfactory results. This may have been due to the fact that gum tragacanth was substituted for India gum which was not readily available. The substitution of digest and lab-lemco for beef infusion broth, also proved unsatisfactory. The medium prepared was violet in colour, and changed little during aerobic

incubation for forty-eight hours, but during anaerobic incubation for the same period its colour changed to a rich violet brown. The addition of sodium taurocholate was not found to be of any advantage. Lipolytic strains of Staphylococcus aureus and albus formed blue colonies as described above, and the blue colour persisted unchanged when the plates were kept at room temperature for ten days. Non-lipolytic strains of Staphylococci showed no blue colour, but Bacterium coli D433, which was not lipolytic when tested by other methods, produced colonies which were faintly tinted blue, after incubation for eighteen hours, although there was no zone of clearing surrounding the colony. This development of colour was considered to be a "false positive" result, and expected to fade rapidly, but it persisted for forty-eight hours in the centre of the streak of growth and only faded gradually when the plates were kept at room temperature for ten days. The colour of the colonies of Bacterium coli D433 was less intense than that of the Staphylococci, and also less so during anaerobic than during aerobic incubation.

The occurrence of false positive reactions had been suggested by Turner (1929), to be due to adsorption of the dye by the bacteria. Similar effects were observed in subsequent work to be described later (Part 1,3), when studying strains of Brucella, Corynebacterium, Mycobacterium and Clostridium. On the theory that some organisms may

produce an endo-lipase which remains bound to the cells, it seems possible that this phenomenon might be due to alteration in the dye adsorbed on to the surface of the cells. This change might be brought about by the activity of endo-lipase bound to the cell surface or liberated locally as the result of autolysis. Some support for this suggestion is given by the observation that, in some cultures, the blue-coloured zone is most apparent in the centre of the colony, or in streak cultures along the centre of the streak, which presumably contains the most ageing cells, and therefore those most likely to produce autolysis. Although there was a clearly defined difference in the intensity and shade of colour of the true and false positive results in this experiment, such a difference may not have occurred in all cases. It is, therefore, conceivable that some of the contradictory observations recorded in published literature, and the difficulty of verifying the claims of some workers to have demonstrated bacterial lipases, may be due to confusion caused by false positive reactions which were not recognised as such at the time when the work was done.

2) FURTHER MODIFICATIONS OF TURNER'S MEDIUM

(1) Inspissated Serum N.B.S. Medium

This medium was developed for two reasons, firstly to see whether serum would help to hold the

fat in suspension and so prevent the formation of surface globules, and secondly to promote the growth of more delicately-growing organisms. The following medium was finally considered fairly satisfactory:

Fat suspension:

Oil (almond or arachis)	27.0 ml.
Hartley broth	180.0 ml.
Gum tragacanth	0.9 gm.

The gum was added to the oil in a large beaker and mixed thoroughly with a thin glass rod. The broth was heated to boiling point and added gradually to the oil and gum with constant stirring, until a creamy suspension was obtained. This was transferred to screw-capped bottles and autoclaved for ten minutes at seven to eight pounds pressure (approximately 112°C), then shaken for ten minutes in the horizontal shaker. The resulting suspension remained stable during storage in the refrigerator for several weeks.

Horse Serum.

The standard product supplied by Burroughs, Wellcome and Company was used. No advantage was found in passing it through a clarifying filter.

Nile Blue sulphate

Nile Blue sulphate (Gurr's)	0.1 gm.
Distilled water	100.0 ml.

The solution was heated gently to dissolve the powder, and stored in the dark.

Complete Medium

The three components were mixed in the following proportions:

Horse serum	79.0%
Fat suspension	20.0%
Dye solution	1.0%

The mixture was shaken automatically for two minutes then kept in a water-bath at 56° C. for a few minutes, or until required. Its reaction was pH 7.4. The plates were warmed in the inspissator and the warm medium poured into them, twenty-five millilitres to a seven centimetre diameter Petri-dish. The temperature of the inspissator could vary from 60 to 90°C. but if it was below 60°C. draughts caused ripples and a broken surface on the plates, if the inspissator was opened before the medium set. The temperature was raised to 90°C. and held there for five minutes, and the inspissator switched off. It was necessary to inspissate the plates with the lids partly open, to avoid water of condensation, which tended to drip on to the medium and mark the surface. The resulting medium was white, with a surface fatty layer, matt, and pale pink in colour.

The organisms to be tested were streaked across the plates, and after incubation those which hydrolysed the fat gave blue colonies which were also surrounded by a region of blue colour. The positive controls were known lipolytic organisms, the Oxford and Mayo strains of Staphylococcus aureus and a strain of Corynebacterium

xerosis known as "B. lipolyticum". The negative controls for the medium were plates without fat, and for the organisms, a strain of Bacterium coli which was found by repeated trial to be non-lipolytic.

Most organisms grew well on this medium, and many experiments were done, but with continued use it was found to be unsatisfactory for the following reasons:

(i) It was technically difficult to prepare, and in unsatisfactory batches the fat tended to separate out.

(ii) The serum varied from one batch to another.

(iii) It was thought that such a high concentration of serum might be inhibitory to some bacteria, or tend to suppress some of the enzyme activity of others.

Before abandoning the medium, however, human fat obtained post mortem was substituted for oil, and incorporated in a suspension in the proportion of 2.0%. Thirty-two strains of bacteria were tested against arachis oil and human fat simultaneously and found to have the same effect on both. Human fat was then replaced by horse fat which is more similar in composition to human fat than that of any other mammal obtainable. Figures for comparison of the fat composition, from Hilditch (1947) are given in Table II.

Table II

Typical Component Acids (Weight %) of Depot Fats of
Different Classes of Animals (Hilditch, 1947) . 105)

Animal	Depot fat	Iodine No.	Saturated Acids Wt. %					Unsaturated acids Wt. %				
			C ₁₄	C ₁₆	C ₁₈	C ₂₀	Total	C ₁₆	Oleic	Octa- deca- noic	C ₂₀₋₂₂	Total
Horse	Abdominal	32.7	-	28.0	5.0	-	33.0	-	50.0	13.0	-	63.0
Sheep	Peri-nephric	43.2	3.0	29.2	21.0	0.4	53.6	2.7	41.7	1.8	0.2	45.8
Human	?	67.4	5.9	25.0	5.8	-	35.1	6.7	45.4	8.2	1.8	64.6
	?	68.9	2.7	24.0	8.4			5.0	46.9	10.2	2.5	

Horse fat was obtained from a retail dealer, cut into small pieces and heated gently in a beaker in an oven, the temperature of which was kept below 100°C. The molten fat was poured off and the tissue residue compressed inside a square of gauze to force out the remaining fat. The extracted fat was stored in small volumes in Universal containers in the refrigerator. Bacteria were found to have a similar action on horse fat to that on the other fats tested.

It was hoped to use pure triglycerides as well as natural fats, but difficulty in obtaining these prevented this investigation.

(2) Serum Fat. N.B.S. Agar.

This medium was used to replace inspissated

serum, owing to the disadvantages of the latter mentioned above, when a good growth of delicately growing organisms was required. The formula given below is the final result of many variations in the proportions of the constituents and it has been found to give very satisfactory results. Several suspending agents were tested in preparing the fat emulsion, Gum Tragacanth (0.25%), absolute alcohol, acetone and a solution (10.0%) of Tween 80 (polyoxyethylene sorbitan monoleate) were all tried before the mechanical emulsifier was obtained. This was a hand-driven domestic model, manufactured by British Emulsifiers Limited, and designated "Cream Maker, Empire Series". The emulsion was prepared as follows:

Melted horse fat	4.0 ml.
Melted aqueous agar, 6.0%	3.0 ml.
Boiling distilled water	17.0 ml.

These were passed through the machine and more hot distilled water added gradually, up to a total of 93.0 ml. A very fine stable emulsion formed, and was sterilised in free steam for ten minutes. The emulsion was reheated immediately before use, and the medium was prepared as follows:

Beef infusion agar	7/ 92.0 ml.
Fat emulsion	7.5 ml.
Horse serum	20.0 %
Nile Blue sulphate	1.5 ml.

The final concentration of fat in this medium was 0.3%. Plates were poured so that a Petri-dish

(diameter 7.0 cm.) contained approximately 25.0 ml., which was found to give the optimum thickness. Plates were dried thoroughly for one hour before use. This medium had the following advantages:

- (i) it supported growth well;
- (ii) it had a matt surface;
- (iii) it had a pale greenish-brown colour when first prepared, which faded to buff during incubation;
- (iv) it showed relatively little variation from one batch to another;
- (v) it was very easily prepared.

Lipolytic organisms growing on this gave the typical appearance characterised by blue colonies surrounded by a zone of colour or clearing.

(3) Nutrient Fat N.B.S. agar.

This medium was used in many experiments and gave satisfactory results with practically all organisms tested. It was prepared from the formula given for serum fat N.B.S. agar in (2) above, omitting the horse serum. The appearance of organisms growing on this medium was very similar to that on medium (2), and is illustrated in Plate I.

(4) Nutrient Fat Agar.

In this medium both horse serum and Nile Blue sulphate were omitted. It was developed to avoid the possible toxic effect of Nile Blue sulphate on the growth



Plate I.

The appearance produced by the lipolytic Staph. albus 476 and non-lipolytic Bact. coli D433, growing on nutrient fat N.B.S. agar medium, for forty-eight hours at 37°C.

of some organisms. This, however, did not prove to be an important consideration, as when the media were used in parallel, comparable results were nearly always obtained. The degree of lipolysis was indicated solely by the extent of clearing, which was generally more marked than in the N.B.S.-containing media. The appearances are illustrated in Plate II, and Plate III.

(5) Fluid Media

Fluid media were tried out and were found to have two disadvantages:

- i) the difficulty of keeping the fat in suspension.
- ii) the very poor colour effects obtained.

The medium was prepared in test-tubes. The basis was 10.0 ml. of Lab Lemco broth. To each tube was added Nile Blue sulphate solution (0.05 ml.) and either crude fat (0.1 ml.) or a suspension (0.5 ml. containing 0.1 ml. fat). The tubes were autoclaved, and when cool inoculated with a loopful of a twenty-four hour agar slope culture of the organism to be tested, stoppered with sterilised rubber bungs, and incubated for forty-eight hours at 37°C. in a rotating mixer.

After incubation most of the fat separated out on the surface in both series. Only a very slight blue tinting of the surface layer could be seen in some of the tubes containing Staphylococcus aureus Mayo; none was seen in the coliform controls.



Plate II.

The appearance of the lipolytic Staph. aureus W3 and the non-lipolytic Bact. coli D 433 growing on nutrient fat agar without N.B.S. The lipolytic activity of the Staphylococcus is shown by the wide zone of clearing in the medium surrounding the growth. The coliform bacillus has produced no change in the medium.

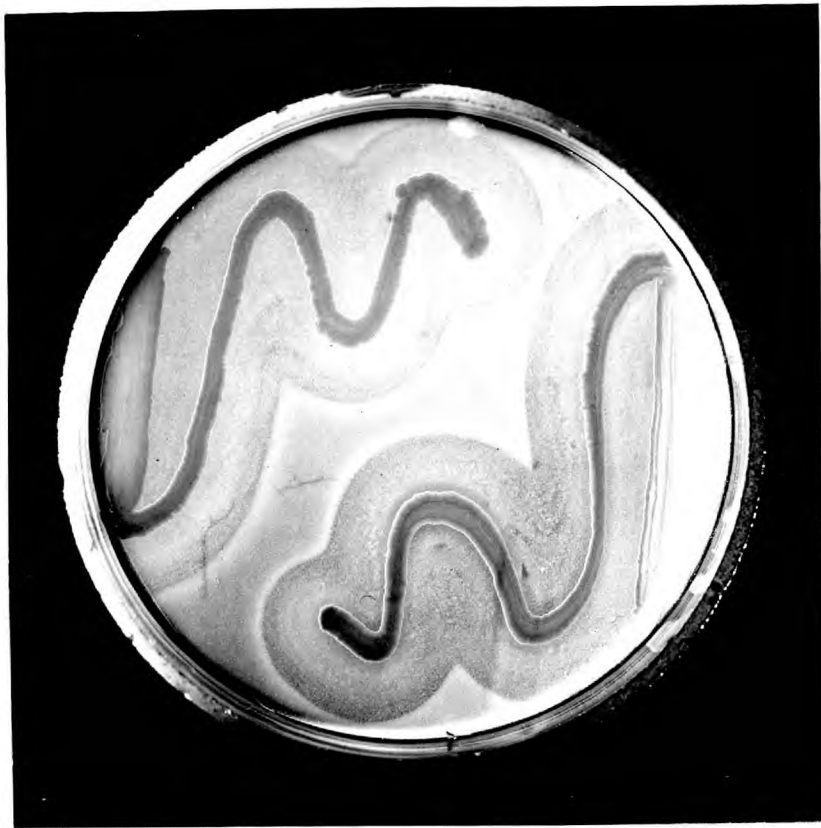


Plate III

The zones of activity produced by two strains of Staph. aureus growing on nutrient fat agar medium.

(6) Inorganic Medium

For further investigation of the lipolytic activity of the Staphylococci, a medium of known composition was used. A basal solution was prepared as follows:

Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.7 gm.
Sodium chloride (Na Cl.)	1.0 "
Ammonium chloride ($\text{NH}_4 \text{Cl}$)	4.0 "
Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.03 "
Buffer solution (KH_2PO_4 M/5)	30.0 ml.
" " (Na_2HPO_4 M/5)	320.0 "
Distilled water to	1,000.0 "

The solution was divided into portions of 100.0 ml. in screw-capped bottles and autoclaved for ten minutes at ten pounds pressure. The reaction was then pH 7.2. Bacterium coli grew readily in this medium when incubated at 37°C . overnight. The addition of yeast extract (Gye, 0.5%) enabled the medium to support the growth of Staphylococci poorly, but if glucose (0.5%) was added as well as the yeast extract, good growth of Staphylococci was obtained. If fat emulsion and Nile Blue sulphate were added, known lipolytic strains of Staphylococci formed blue colonies, and, without the dye, clear haloes round the colonies. In several batches sodium taurocholate (0.5%) was added, but it did not markedly increase the clearing effects (Turner, 1929).

This medium proved useful for organisms which could grow in the basal portion and was reserved for

such strains, while a more complex synthetic medium was developed for Staphylococci.

(7) Synthetic Medium for Staphylococci.

This medium was used during the second part of the work. It was based on the amino acid medium of Fildes and co-workers (1936) as modified by Fildes and Richardson (1937), and was prepared as follows:

Basal Medium:

<u>Nutrient</u>	<u>Weight or Volume.</u>	<u>Concentration in Final Medium.</u>
Potassium dihydrogen phosphate	4.5 gm.	
Distilled water	550.0 ml.	
Sodium Hydroxide N/1	26.0 "	
s-alanine	0.12 gm.	N/1,500
s-valine	0.15 "	"
s-leucine	0.17 "	"
s-glycine	0.05 "	"
l-proline	0.07 "	"
l-oxypoline	(not available)	
s-aspartic acid	0.18 "	"
d-glutamic acid	0.09 "	"
s-phenylalanine	0.08 "	N/4,000
l-tyrosine	0.05 "	"
d-arginine	0.05 "	"
l-histidine	0.05 "	"
s-lysine hydrochloride	0.09 "	"

(s- denotes the synthetic product).

The amino acids were dissolved, the reaction adjusted to pH 7.4 and the volume brought to 600.0 ml. The solution was then distributed in volumes of 60.0 ml.

in screw-capped bottles and sterilised by autoclaving for ten minutes at ten pounds pressure.

The remaining constituents were prepared and stored separately, being added aseptically to give multiples of 100.0 ml. of complete medium.

Ferrous ammonium sulphate

(M/500 in M/50 hydrochloric acid, autoclaved) 2.5 ml.

Magnesium sulphate

(M/60, autoclaved) 1.0 "

Sodium nitrate

(M/5, autoclaved) 1.0 "

s-aneurin

(M/2,000 in M/1,000 hydrochloric acid, autoclaved, diluted 1/100) 2.0 "

s-nicotinic acid

(M/200, autoclaved, diluted 1/10) 2.0 "

s-methionine

(M/100, filtered) 2.0 "

l-tryptophane

(M/100, autoclaved) 1.0 "

l-cystine

(M/100 in M/10 hydrochloric acid, filtered) 2.0 "

Sodium hydroxide

(M/5, autoclaved) 1.0 "

Glucose

(M/2, filtered) 2.5 "

Distilled water

23.0 "

Fat was added to the medium in the proportion of 75.0 ml. of emulsion to the litre of medium, giving

approximately 0.3% fat. If the medium was to be made solid, the 23.0 ml. of water was replaced by 23.0 ml. of 6.0% aqueous agar fibre solution.

The formation of clear zones around colonies was the usual indicator of lipolysis on this medium. When used simultaneously with nutrient fat agar agreement between results was good, although lipolytic effects on the synthetic medium tended to be fainter than those on nutrient medium.

(8) Maintenance of Stock Cultures.

All strains of micro-organisms for which nutrient agar is an adequate medium were maintained on agar slopes. For more exacting strains blood or serum media were used. Whenever possible strains being investigated were dried for preservation (Stamp, 1947). Later in the work milk was used in the hope that because of its fat content it would preserve the lipolytic activity of some strains which were losing it after prolonged subculture over nearly three years, but the milk was not found to have any such advantage. When using synthetic medium, stock strains were kept in fluid cultures of synthetic medium during the period of the investigation.

3) SYSTEMATIC INVESTIGATION OF GENERA

The first two experiments were done on inspissated serum medium, and later repeated on serum fat N.B.S. agar (Medium (2)). The organisms tested were a miscellaneous collection of dried stock cultures, including eighty strains of the *Corynebacteria*. With very few exceptions the results obtained on serum fat agar verified those on inspissated serum. All subsequent experiments in Part I were done on serum and nutrient fat N.B.S. agar, (Media (2) and (3)).

In all experiments young cultures, (eighteen to twenty-four hours), were used, and tested for lipolytic activity by streaking across the fat plates. Every plate was inoculated with a central diametrical streak of a known lipolytic organism, to act as control. The Mayo strain of Staph. aureus was used at first, and later replaced by a more active strain of Staph. albus from a throat-swab, and designated "476". Stock cultures of Staph. albus 476 retained their lipolytic activity undiminished by weekly subculture for almost three years, when the lipolytic power suddenly ceased and could not be restored. All organisms being investigated were cultured on two different batches of fat medium, before a definite result was accepted. Anaerobic cultures were put up when necessary. Incubation time was usually forty-eight hours at 37°C. followed by three to seven days at room temperature.

Preliminary Study of a Few Strains from a Large Number of Genera.

The organisms included in this test were, with the exception of the genera *Neisseria* and *Haemophilus*, subcultured from dried stock cultures. Although the numbers of each genus were too small to be statistically significant, the results were considered as an indication of those genera likely to show lipolytic activity by this method. The results are given in Table III.

More detailed Study of Certain Genera.

1) *Corynebacterium diphtheriae*.

These strains were subcultured from the dried state, the gelatin discs melted in broth and subcultured on to blood-agar and Downie plates, and on to inspissated serum and serum fat agar. Eighty strains were tested:

<u><i>C. diphtheriae</i></u> <i>gravis</i> types	42
inter types	18
<i>mitis</i> types	20

A proportion of these strains were found to be virulent as estimated by the intradermal injection of guinea pigs (Mackie and McCartney, 1948).

On inspissated serum medium (Part I. 2,1), four *gravis*, five inter and two *mitis* strains produced colonies with a blue colour after forty-eight hours of incubation, which faded on continued incubation up to ninety-six hours. It seemed possible that this development of blue colour might be due to lipase production, but the result was not

TABLE III

<u>Genus</u>	<u>No. of Strains Tested</u>	<u>Arachis oil</u>	<u>Lipolysis</u> <u>Human and Horse Fat</u>
Clostridium	8	-	-
Bacillus	7	-	-
Streptococcus			
pyogenes	5	-	-
" viridans	1	-	-
" faecalis	1	-	-
" pneumoniae	3	-	-
" anaerobic	1	+	+
Staphylococcus			
aureus	8	+	+++ in all.
" albus	5	+	+++ " "
Micrococcus	3	+	+ in two
Sarcina	2	-	-
Neisseria	4	-	-
Coliform	12	-	-
Salmonella	5	-	-
Shigella	3	-	-
Vibrio	6	-	trace
Haemophilus	3	-	-
Pasteurella	4	-	-
Brucella	3	Colonies pale green in colour; ? due to adsorption of dye.	
Chromobacterium	1	-	-
Mycobacterium	1	-	-
Corynebacterium	10	+	+ in one (B. lipolyticum)
Yeast	1	-	-

confirmed using serum fat agar medium. It was therefore concluded to be a false positive reaction, such as has been described earlier (Part 1, 1).

Diphtheroids.

One hundred and thirty strains of dried stock cultures of diphtheroids, originally collected from routine laboratory plate cultures, were tested for the production of diffusible lipase on serum fat agar. The strains were grouped according to their fermentation reactions in glucose, saccharose and dextrin (Mackie and McCartney, 1948).

<u>C. xerosis</u>	49 strains
<u>C. hofmanni</u>	26 strains
Other types	55 strains

All strains grew well, but none produced a diffusible lipase.

The negative results observed in this genus agree with those of earlier workers, notably Michaelis and Nakahara (1923).

2) Bacterium coli.

Many of the organisms in this group had been isolated from cultures of urine from patients with cystitis, in order to include strains for which there is some evidence of pathogenicity. Some normal faecal strains were included in the test.

Michaelis and Nakahara (1923) claimed to have

isolated lipolytic strains of Bact. coli from the faeces of breast-fed infants. They used a chemical extraction method, which probably liberated an endo-enzyme.

Attempts were made to determine whether any similar strains could produce diffusible lipase on nutrient fat agar medium. More recent work by Taylor, Powell and Wright (1949) showed that specific serological types of Bact. coli are associated with infantile diarrhoea. A number of strains were, therefore, isolated from the faeces of premature and very young infants in Hammersmith Hospital, during and after an outbreak of infantile diarrhoea. Some of these strains were collected by Dr. D.R. Barry and a subculture of the strain D433 was kindly given by Dr. Joan Taylor. Among the strains collected by Dr. Barry, sixteen out of sixty-two were agglutinated by the serum of a rabbit immunised against the strain D433, and therefore were concluded to belong to the serological type believed to be associated in some way with infantile diarrhoea.

All strains were identified by means of their fermentation reactions in glucose, lactose, dulcitol, mannitol, saccharose and salicin and their ability to produce indole, and give the Methyl Red and Voges-Proskauer Reactions.

The strains were tested for the production of diffusible lipase on serum and nutrient fat agar, with and without the addition of Nile Blue Sulphate (media 2, 3 and 4), and on simple inorganic medium (medium 6). No evidence

of diffusible lipase production was found.

When the strains were tested in duplicate, one set of plates being incubated anaerobically, there was again no evidence of lipase production. Cultures of the *Staphylococcus* control incubated anaerobically were similar to those grown under aerobic conditions.

The strains of Bact. coli tested were grouped as in Table IV.

Table IV.

Strains of Coliform Bacilli Tested for Production
of Diffusible Lipase.

Source of strain	No. of strains tested	Lactose fermenters	Non-lactose fermenters.
Cases of cystitis	135	121	14
Faeces of premature infants	88	88	none
Normal faeces	25	20	5

3) Salmonella enteritidis.

Subcultures from forty-one strains of Salm. enteritidis were kindly given by Dr. W. Hayes, and tested for lipolytic activity on nutrient fat agar. The strains were as follows:

<u>Salmon. enteritidis</u>	Jena	8 strains
	Danzig	1 strain
	Essen	2 strains
	Chaco	30 strains (included 5 original strains isolated from men in the Chaco war.)

The test was repeated on a second batch of medium, but no evidence of diffusible lipase was found in either test. As no claims to have demonstrated diffusible lipases from the Salmonella have been found, this negative result was considered to verify those of earlier workers.

3a) Saprophytic Gram-negative Bacteria.

Strains of Gram-negative bacilli isolated from water were examined to determine whether lipolytic activity was more characteristic of saprophytic Gram-negative bacilli, as had been reported by Collins and Hammer (1934).

A sample of water was taken from the River Thames near Putney Bridge and from this sample twelve strains of Gram-negative bacteria were isolated. Three were found to be lipolytic. They were non-lactose fermenters and biochemically inert when cultured in fourteen different sugar media and litmus milk and on gelatin, and the indole, Methyl-Red and Voges-Proskauer reactions were negative. Two of the three strains utilised Koser's citrate medium. Their optimal temperature of growth was about 22°C.

It would appear, therefore, that amongst the Gram-negative bacilli, diffusible lipase is produced only by strains which are biochemically relatively inactive. The reverse observation was made among the Staphylococci studied later (Part II,2). Lipase production may also be related to the temperature at which optimal growth occurs, an aspect which has been studied in much greater detail in a later section (Part III).

4) Mycobacterium tuberculosis.

For this group serum fat agar and serum fat N.B.S. agar media were reinforced with glycerol (5.0%). The medium was sloped in Universal containers. The cultures were incubated at 37°C. for four months, being examined at two-weekly intervals.

One hundred strains of Myco.tuberculosis, human type, were collected from cultures on Lowenstein-Jensen medium, grown from specimens sent to the laboratory for routine investigation. Eight other strains from different sources were included in the test.

On serum fat agar, twenty-strains (18.5%), developed blue-coloured colonies, between the fourth and eighth week of incubation, and the blue colour persisted until the cultures were discarded. All blue colonies were filmed, and the films stained by Ziehl-Neelsen's method. None of the one hundred and sixteen strains tested showed any zones of clearing on nutrient fat agar medium. The observations are shown in Table V.

Table V.
Mycobacteria Tested for the Production
of Diffusible Lipase.

No. of strains tested.	Source	Type	No. of strains showing Clear zones Blue colour.	
58	Sputum	Human	0	11
22	Laryngeal swabs	Human	0	3
5	Pus	Human 3 Bovine 2	0	1 0
8	Urine	Human	0	2
3	Gastric juice	Human	0	1
4	Pleural fluid	Human	0	0
3	N.C.T.C.	Bovine	0	1
1	N.C.T.C.	B.C.G.	0	0
1	Stock	H 37 Human	0	0
1	Stock	H 37 Ra Human	0	0
1	N.C.T.C.	Saprophyte	0	0
1	N.C.T.C.	<u>M. phlei</u>	0	0

Almost all the strains grew well on both media, but

a few grew poorly. Since no evidence of lipase production was found, as estimated by clearing of the medium, it was concluded that the proportion of strains developing blue colonies were giving false positive results, such as have been described earlier. (Part 1, 1)..

5) Clostridium welchii.

Cl. welchii was more extensively studied than any other species in this group, because of its ability to produce lecithinase, to which it seemed lipolytic activity might be related. Five strains were obtained from dried stock cultures, one of which was designated 6719, and was known to be highly toxigenic and lethal to rabbits. Sixty-nine strains were isolated from specimens of faeces, and identified as Cl. welchii by their colony formation, the production of a "stormy clot" in litmus milk, and the Nagler reaction (Nagler, 1939; Hayward, 1941, 1943). Films were made of doubtful strains. Single strains of several other species of the Clostridia were also tested. The observations were as in Table VI.

Further tests were carried out to confirm that Cl. welchii did not produce a diffusible lipase. The medium was modified by

- 1) heating the horse serum before adding it to the medium, and replacing it by Fildes' extract;
- 2) varying the amount of dye;
- 3) varying the pH.

No positive results were obtained.

Table VI

Clostridia Tested for the Production of Diffusible Lipase
on Nutrient Fat Agar

Species	Source	No. of Strains	Stormy clot	Nagler reaction	Lipolysis
<i>Cl. welchii</i>	Stock	5	+	+	-
<i>Cl. welchii</i>	Faeces	59	+	+	- *
Unidentified Clostridia	Faeces	10	+ -	+ -	-
<i>Cl. oedematiens</i>	Stock	1	/	/	+++
<i>Cl. Oedematiens Albiston</i>	Government Research Laboratory, Porton	1	/	/	-
<i>Cl. botulinum</i>	Stock	1	/	/	+++
<i>Cl. botulinum</i> Type A. 4587	Government Research Laboratory, Porton	1	/	/	+++
Type B, BEANS	Government Research Laboratory, Porton	1	/	/	+++
Type B, OKRA	" "	1	/	/	+++
Type E, NANAINO	" "	1	/	/	-
<i>Cl. histolyticum</i>	Stock	1	/	/	-
<i>Cl. tetani</i>	Stock	1	/	/	-
<i>Cl. septique</i>	Stock	1	/	/	-
<i>Cl. tetanomorphum</i>	Routine laboratory	1	/	/	-
<i>Staph. albus</i> 476	Control	1	/	/	+++

* Abnormal greenish-yellow colour in 58 strains. No coloured zone in the medium.

The appearance of these colonies was somewhat similar to that observed with strains of *Brucella* (Table III. p. 64a), which were reported as false positives, and described earlier (Part 1,1).

Attempts were next made to produce diffusible lipase from these strains by growing under different conditions, namely in a fluid medium such as Robertson's cooked meat medium. Forty-eight hour cultures were centrifuged, and the supernatant fluid distributed into porcelain cylinders embedded in nutrient fat agar. No clearing of the medium appeared round the cylinders.

6) Miscellaneous Anaerobes.

A search was made for anaerobic lipolytic

* Nasal
Buccal
Throat
Cervical
Vaginal

* bacteria, by rubbing various swabs across the surface of fat agar plates in a single streak. The plates were incubated at 37°C. for forty-eight hours, and kept at room temperature for five days thereafter, and examined for colonies coloured blue or surrounded by a blue zone. All such colonies were subcultured aerobically, filmed and classified.

No lipolytic obligate anaerobes were found, all positive colonies proving to be either Staphylococci or Micrococci, and in two cases, Gram-negative cocco-bacilli. Twenty-four lipolytic strains were isolated from a total of one hundred and twenty-nine swabs examined.

In addition, six strains of anaerobic Gram-negative bacilli from abdominal wounds and nine strains of Bacteroides (kindly given by Dr. G.I.M. Ross), were examined. For the Bacteroides laked blood (0.5%) was added to the medium. None of these organisms was found to have any lipolytic

activity.

7) Gram-Positive Cocci.

Strains were subcultured from stock cultures and from plate cultures of swabs, sputum, pus, faeces, urine and blood cultures. All Staphylococci were tested for the production of coagulase (Mackie and McCartney, 1948). The organisms were tested for the production of diffusible lipase on serum and/or nutrient fat agar, incubated aerobically at 37°C. for forty-eight hours, and kept at room temperature for several days thereafter. The observations have been grouped in Table VII.

In this group, and particularly among the Staphylococci, differences in the degree of lipolysis were very clearly marked. The effects observed were similar to those described in the section on Turner's work (Part I, 1), namely variations in the depth of blue colour of the colonies and in the width of the surrounding zone of clearing. In the majority of strains tested the lipolysis was uniform, all colonies on the plate showing similar activity.

Twenty-four and forty-eight hour culture filtrates of several strains were examined for lipolytic activity, using the plate and cylinder technique, but all gave negative results. This was probably due to the period and other conditions of growth, in view of the positive results to be described in a later section. (Part IV, 2).

Table VII

Gram-positive Cocci Tested for the Production of
Diffusible Lipase.

Organism	No. of strains Tested.	No. of strains lipolytic		No. of strains non-lipolytic.	
		Coag. Pos.	Coag. neg.	Coag. Pos.	Coag. neg.
Staph. aureus	48	39	2	5	2
Staph. albus	38	11	18	3	6
Staph. citreus	2	1	0	0	1
Micrococcus	13	0	10	0	3
Sarcina	1	Ø	Ø	Ø	1
Strep. pyogenes	38	Ø	Ø	Ø	38
Strep. viridans	4	Ø	Ø	Ø	4
Strep. faecalis	1	Ø	Ø	Ø	1
Strep. pneumoniae	9	Ø	Ø	Ø	Ø
Non-haemolytic streptococci	5	Ø	Ø	Ø	Ø
Total	159	51	30	8	12 70

	<u>No. tested.</u>	<u>Proportion lipolytic</u>
<u>Staph. aureus</u>	48	41 (85.4%)
<u>Staph. albus</u>	38	29 (76.3%)

8) Airborne Micro-organisms.

A number of plates which were kept at room temperature for periods of several weeks were found to have developed contaminant colonies. A total of thirty-three colonies were examined. Of these, nineteen proved to be fungi, none of which gave a positive result. Of the fourteen bacterial colonies examined, two were lipolytic, and both of these proved to be Gram-positive cocci.

Summary and Conclusions of Part I.

The work reported in this section has shown that the production of diffusible exo-lipases is not characteristic of pathogenic micro-organisms, and is confined to one or two species only. In these, as for example the Staphylococci, there did not appear to be any correlation between lipase production and specific biological characteristics such as haemolysin and coagulase production, since it was demonstrated in a number of albus strains and Micrococci, and also in some coagulase-positive strains of Staph. aureus, presumed to be pathogenic. On the other hand, it was more commonly found in strains with marked fermentative ability than in weakly fermentative strains. The reverse appears to be the case among the Gram-negative bacilli, in which lipolysis was demonstrated only in strains of saprophytic origin, showing little or no fermentative ability.

The techniques employed were only suited to the detection of lipases capable of diffusing from the actively growing cells. This may explain in part the failure to demonstrate lipolytic activity in some species which, in the hands of other workers, using chemical extraction methods, have given positive results. It is noteworthy that only two of these species have given what have been referred to as "false positive results", in which the blue or green colouration produced by the

action of fatty-acids on the Nile Blue sulphate was very slight and confined to the growth. This may be due to the lipolytic activity of enzymes bound to the cells, and liberated only to a slight extent as the result of autolysis.

Part II.

FURTHER INVESTIGATION OF STAPHYLOCOCCI PRODUCING LIPASE

In this section a study was made of lipase production in relation to the other metabolic activities of Staphylococci, including pigment production, general biochemical activity, the probable production of enterotoxin and the production of haemolysis on blood-agar plates. In addition, individual strains of lipolytic and non-lipolytic Staphylococci were investigated for the presence of mutants, and attempts were made to develop or increase the number of these mutants by successive subculture in media with and without fat. The effects of incorporating fat in sub-optimal media on the degree of growth were studied, and attempts were made to determine whether any metabolites were essential for lipase production.

Before proceeding with these experiments, a comparison was made of the solid medium technique for demonstrating lipolysis, which had been used previously and fluid medium techniques. These included titration of acidity produced in fat-containing broth, as described by Trussell and Weed (1937).

1) Comparison of Solid and Fluid Medium Techniques.

The solid medium technique used was that

described in Part I (7), using nutrient fat agar. The fluid medium technique was as follows:

Beef infusion broth in aliquots of 50.0 ml. was sterilised in conical flasks, capacity 250.0 ml. To half of these was added the aqueous agar suspension of fat already described, to give a final concentration of 0.3 per cent fat. The flasks were inoculated in pairs, one with fat and one without, with 0.1 ml. of a twenty-four hour infusion broth culture of the organisms to be tested, and incubated at 37°C in the mechanical shaker. Trussell and Weed (1937) found that twenty-four hours of incubation in a shaking machine was sufficient for completion of lipolysis, but in the conditions obtaining in this experiment, forty-eight hours was found to be the optimum time.

After incubation the flasks were diluted with an equal volume, 50.0 ml. of distilled water, and the indicator, 0.5 ml. bromo-thymol blue added to each flask. The cultures without fat turned green in colour, while those with fat, in which the fat had been hydrolysed turned yellowish, owing to increase in acidity following the liberation of fatty-acids. The cultures with fat in which the fat had not been hydrolysed, were the same green colour as the controls. The cultures showing hydrolysis were then titrated with sodium hydroxide (0.1 N), until the colour matched that of the controls. Examples of the figures obtained in the titrations and their correlation with the results on solid medium are given in Table VIII.

Table VIII

Micrococcus	Lipolysis as indicated on solid medium.	Lipolysis as indicated by titration: millilitres of N/10 sodium hydroxide
21	+++	6.3
31	+++	7.5
M7	+++	4.7
17	++	2.6
22	++	2.1
58	++	2.7
55	+	nil
105	+	nil
141	+	nil
Negative 96	-	nil
controls 2	-	nil

It was found that the results of the two methods, on solid and in fluid medium, agreed well in most cases. When the organism was actively lipolytic both methods seemed fairly sensitive and equally suitable, but in cases where the organism was only slightly lipolytic the development of zones of clearing or of colour was more easily recognised than were slight changes in the shade of the indicator in fluid medium. For this reason the use of solid media seemed preferable.

2) Lipase Production in Relation to Pigment Production.

Reference has already been made (Part 1, 7, Table VII), to the fact that 85.4% of aureus strains

and 76.3 % of albus strains of Staphylococci were lipolytic. The albus strains should, however, be divided into those which have a general biochemical activity equal to that of aureus strains, and may be regarded as albus variants of aureus strains, and those with weaker powers of fermentation, comprising the heterogeneous group of white Staphylococci and the so-called Micrococci.

Twelve strains of Staph. aureus were selected, having a high degree of biochemical activity. They fermented glucose, lactose, saccharose, mannitol, maltose and glycerol, but not dulcitol, inulin, raffinose nor salicin. They produced acid in litmus milk, liquefied gelatin within six days and gave a positive Methyl Red reaction and a variable Voges-Proskauer reaction. Nine of these twelve strains (75.0%) were found to be lipolytic.

Six strains of Staph. albus having similar fermentation reactions, were tested for lipolytic activity, and of these four (66.6%) were lipolytic.

In the relatively small number of strains studied, lipase production would, therefore, appear to be unrelated to pigment production.

3) Lipase Production in Relation to General Biochemical Activity

Seven strains of Staph. albus having much weaker fermentative ability, were compared with the albus strains in section (2) above. These weakly fermenting strains

fermented glucose constantly, lactose, saccharose and mannitol in a moderate proportion of cases, and maltose and glycerol rarely. All produced acid in litmus milk, and a few liquefied gelatin and gave positive Methyl Red and Voges-Proskauer reactions. None of these was found to be lipolytic.

It would appear, therefore, as has been suggested from previous work (Part I, 7), that lipolysis is commoner among strains of Gram-positive cocci with a high degree of metabolic activity.

4) The Investigation of Some Strains of Staphylococci Associated with Outbreaks of Food-Poisoning for Lipolytic Activity.

It is well known that certain strains of Staphylococci are able to produce a heat-stable enterotoxin, which is responsible for a considerable proportion of cases of "toxic" type food poisoning, (Burnet, 1930; Parish and Clark, 1932).

The ability to produce enterotoxin has not been shown to be related to any other biological characteristic of the Staphylococci, although Slanetz (1942) suggested that enterotoxin and beta-haemolysin might be identical, Woolpert and Dack (1933) had concluded that enterotoxin was distinct from the other toxins of the Staphylococci. It seemed of interest, therefore, to study the lipolytic activity of strains of Staphylococci isolated from staphylococcal food-poisoning material, to determine whether any specific

relationship existed between enterotoxin production and lipolytic activity.

The difficulty of proving the presence of enterotoxin in culture filtrates, by methods such as the intraperitoneal injection of kittens, (Dolman, Wilson and Cockcroft, 1936; and Davison, Dack and Cary, 1938) and the feeding tests of Jones and Lochhead (1936) and Fulton (1936), made it necessary to rely on presumptive evidence for its production. It was, therefore, assumed that, as the organisms had been isolated from material associated with cases of food-poisoning, they had produced enterotoxin. It was realised, however, that this assumption was not wholly justifiable.

Twenty-five strains of Staph. aureus associated with outbreaks of food-poisoning were kindly supplied by Dr. R.E.O. Williams of the Central Public Health Laboratory, Colindale, London, and by Dr. J. Marks of the Public Health Laboratory, Cardiff. Although strains of Staph. albus have been claimed to produce enterotoxin, (Stirtar and Jordan, 1935), no albus strains were included in this test.

These strains were tested for the production of diffusible lipase, along with an equal number of strains of comparable biochemical activity from routine pathological sources, and from samples of innocuous foodstuffs, isolated by the method of Maitland and Martyn (1948), using cooked meat medium containing

10.0 per cent sodium chloride. Lipase production on solid medium was verified by the titration method described in Part II, 1. The biochemical activity of all strains was tested as described in Part II, 2, using glucose, lactose, saccharose, mannitol, maltose and glycerol.

All the strains associated with food-poisoning were found to be actively lipolytic, while only twelve of those of comparable fermentative ability from innocuous foodstuffs were lipolytic. But, as nearly all the control strains obtained from other pathological material, having a similar high degree of biochemical activity, were also lipolytic, no deduction could be made as to any specific relationship between lipase and enterotoxin production.

A comparison of the two groups of organisms from pathological material is set out in Table IX.

Table IX.

Comparison of the Lipolytic Activity of Staphylococci
From Outbreaks of Food Poisoning with that of Similar
Strains Isolated from Routine Pathological Specimens.

Source of Staphylococci	No. of strains tested	No. of sugars fermented	No. of strains lipolytic	No. producing coagulose	No. liquefying gelatin	No. haemolytic on blood-agar.
Food-poisoning material	25	6	25	25	25	25
Pathological specimens	20 (coagulose positive)	6	19	20	20	9
	5 (coagulose negative)	6	1	5	0	2

5) The Relationship Between Lipase Production and Haemolysis On Blood-Agar Plates:- The Possible Interrelationship of Lipase and Haemolysin.

The lysis of red blood corpuscles of different animal species can be brought about by many substances of different chemical structure. These vary from simple molecules such as phenol, to the complex molecules of vegetable alkaloids, animal poisons such as snake venom and the bacterial toxins, but in all cases, the appearance of the resulting haemolysis is the same. In view of this variety in chemical structure, it seems possible that many substances produced by bacteria might be able to lyse red blood corpuscles, in addition to the specific substances called haemolysins. It was suggested by Williams and Harper (1947) that lipase is associated with the delta-haemolysin of *Staphylococci*. The lipid content of human, bovine, sheep and equine red blood corpuscles has been demonstrated to be similar by a group of workers (Erickson, Williams, Bernstein, Arvin, Jones and Macy, 1938) and shown to consist of phospholipid 60.0 per cent (50.0% cephalin), free cholesterol 30.0 per cent and cholesterol esters and neutral fat 10.0 per cent. If lipase does produce haemolysis by breaking down the lipids in the red cell envelope, then lipolytic strains could be expected to be consistently haemolytic.

Parallel tests for haemolysin production on blood agar plates and lipase production on nutrient fat agar were, therefore, carried out to test this hypothesis.

It was found that nineteen out of seventy-two lipolytic strains of Staphylococci were non-haemolytic, which suggested that lipase is not concerned in bringing about lysis of red blood cells in normal circumstances. It is possible, however, that lipase might produce haemolysis in abnormal circumstances, in which excess of fat is present in the blood-agar plate, by indirect means, that is, as the result of free fatty-acids produced laking the red blood cells. This possibility was tested by carrying out parallel tests for the development of zones of haemolysis on blood-agar plates, and on blood-agar plates containing fat (0.3%), with seventy-two lipolytic strains of Staphylococci. Two out of fifty-three strains were found to produce beta-haemolysis on fat-containing plates, but not on ordinary blood-agar. This suggested that when fat is present, lipolytic but ordinarily non-haemolytic strains may produce a haemolytic zone, probably due to lysis of the red blood cells by the free fatty-acids liberated, (as was put forward by Christie and Graydon, 1941). This zone of haemolysis is in no way different from that produced on fat-free blood-agar.

6) The Occurrence of Mutants in Lipolytic and Non-Lipolytic Strains of Staphylococci.

The existence of lipolytic mutants in apparently non-lipolytic strains and vice-versa, was investigated.

(i) Demonstration of mutants by plate culture method.

Strains were examined for the presence of lipolytic and non-lipolytic variants by growing for twenty-four hours in infusion broth, with and without fat, and plating out to obtain single colonies. (This was done by diluting the culture and delivering drops of $\frac{1}{2} \times 1/10^8$ dilution on to the surface of nutrient fat agar plates, by means of a "50" dropping pipette) . After incubation at 37°C. for forty-eight hours, the plates were examined for mutant colonies. When these were found, they were subcultured through fluid medium to nutrient fat agar plates, to determine whether the variation would remain constant throughout subsequent subcultures.

Four strains were investigated, two of which were lipolytic, Staph. albus 29 and 476, and two non-lipolytic, Staph. albus 59 and 96.

The two lipolytic strains were found to remain consistently lipolytic, all colonies appearing to have the same degree of lipoclastic activity. Single colonies subcultured through broth, with and without fat, and back to nutrient fat agar, remained fully lipolytic through ten subcultures.

The two non-lipolytic strains produced a small number of lipolytic variants. Staph. albus 59 produced 2 variants in 23,000 colonies, one grown in nutrient broth with fat, the other in nutrient broth without fat.

Both of these produced golden pigment and retained their lipolytic activity unimpaired during transfer through five subcultures. Staph. albus 96 produced 3 lipolytic variants among 39,000 colonies, one from a culture in nutrient broth with fat, and two from cultures in nutrient broth without fat. Only one of these variants produced golden pigment. Its sugar fermentation reactions were the same as those of the original strain. All three variants retained their lipolytic power unaltered through five successive subcultures, of each of which approximately one thousand colonies were examined.

It would thus appear on the basis of the examination of a very limited number of strains, that lipolytic strains are more stable, and do not produce mutants with the same ease as non-lipolytic strains.

(ii) Selection of mutants by "training" by a tube culture method.

Attempts were made to develop or increase the frequency of mutants by prolonged subculture in media with or without fat. One strain in which no mutants had been observed in the previous experiment (i), was used, namely the lipolytic strain Staph. albus 476, and the non-lipolytic strain Staph. albus 23. In addition the two non-lipolytic strains of Staph. albus 59 and 96, which had been shown to possess rare lipolytic mutants, were submitted to the same procedure.

Full strength inorganic medium was used for this test. A duplicate series of test-tubes of medium, one of which contained 0.3 per cent fat, was used for each strain tested. Subcultures were made daily and every sixth subculture was plated out on nutrient fat agar and the colonies observed for lipolysis.

The lipolytic strain Staph. albus 476 continued to produce fully lipolytic colonies through fifty-five subcultures in synthetic medium containing fat. In the medium without fat, the same result was obtained through forty-five subcultures. During a further ten subcultures, however, individual colonies showed an equal but gradually decreasing degree of lipolysis, until lipase production finally ceased. The strain was thereafter consistently non-lipolytic through twelve subcultures. The last subculture was then inoculated back into medium containing fat, to determine if the process could be reversed, and the power to produce lipolytic colonies restored, but after seventy-two subcultures the organism was still completely non-lipolytic. This loss of lipolytic activity would appear to be due to a different mechanism from that brought about by the selection of mutants.

Staph. albus 23 was passed through thirty subcultures without showing any evidence of the development of lipolytic colonies.

There is no evidence, therefore, from experiments

with these two strains, that mutant colonies can be developed by subculture when they are not evident in the parent culture.

On the other hand, after subculturing the non-lipolytic Staph. albus 59 through twenty-six subcultures in medium containing fat, 100.0 per cent lipolytic colonies were produced on plating out. After a further seven subcultures in fat-containing medium, the lipolytic activity of individual colonies was maximal. This lipolytic mutant was then subcultured into medium containing no fat, to determine whether non-lipolytic mutants would be produced. There was no change after eighteen subcultures, but after a further twelve subcultures, lipolytic activity, although similar in individual colonies, showed some decrease, and was entirely absent by the fortieth subculture.

Staph. albus 96, the other strain which showed rare lipolytic mutants in the parent culture, was submitted to the same procedure, but in this case the development of a fully lipolytic culture was not brought about, although the process was continued through seventy-one cultures.

The first series of experiments showed that, among the very limited number of strains studied, lipolytic strains of Staphylococci may be more stable than non-lipolytic ones. No non-lipolytic variants were found in the two lipolytic strains, but the non-

lipolytic strains were found to produce relatively rare lipolytic mutants.

The second series showed that these rare lipolytic mutants could be increased in number as the result of subculture in fat-containing media, until the culture appeared to be fully lipolytic:- a process of selection discussed by Dubos (1940). Lipolytic and non-lipolytic strains which appear to have no mutants in the parent culture could not, however, be induced to produce any by this method. Lipolytic strains subcultured for relatively long periods (eighteen to thirty-six subcultures) in medium without fat, underwent a gradual loss of lipolytic activity.

7) The Effect of the Addition of Fat to Diluted Medium on the Growth of Staphylococci.

These experiments were based on the expectation that, when growing in deficient medium, organisms which do not normally act upon fat might be induced to utilise it as a source of fermentable carbohydrate. The first experiments were done by growing the test organisms in infusion broth diluted 1 in 10 with distilled water. To half of these tubes fat suspension was added to give a final concentration of 0.3 per cent fat. The organisms were subcultured daily through both series ^{of} tubes, with and without fat, in parallel, and opacity estimations carried out, along with viable counts on agar plates, made

by the dilution method. This latter technique was introduced owing to the difficulty of estimating differences in opacity, because of the slight turbidity in the medium caused by the fat.

No difference was observed in the density of growth in cultures with and without fat, the growth in all cases being equally profuse. Likewise, no significant difference in viable counts was found.

When the test was repeated using a 1 in 10 dilution of synthetic medium, a similar result was obtained.

It was apparent, therefore, that fat did not help the growth of either the lipolytic or the non-lipolytic strains tested.

8) The Effect of Varying the Constituents of Synthetic Medium on Growth and Lipase Production.

This experiment was designed to determine which, if any, of the constituents of synthetic medium were essential for the production of lipase. The medium used was based on that of Fildes and Richardson (1937), Part I. 2, 6. Since a great number of variations would be possible in an aqueous medium with twenty-four constituents, the experiments were carried out in two groups. In the first, the basal part of the medium was unaltered, and the additional amino-acids omitted in turn. In the second group, the basal part of the

medium was modified, and the additional amino-acids added as for the complete medium. In all modifications the total volume required was made up by the addition of distilled water.

It was found by preliminary experiments that a number of strains would still grow even although several constituents were omitted simultaneously. Supporting evidence was provided by Sargolla (1937), that *Staphylococci* grow and produce enterotoxin in synthetic media in which the number of amino-acids present varied from two to sixteen, provided that glucose was present as a source of carbohydrate. The two amino-acids which proved adequate were arginine and cystine. In view of this evidence, and because they were so actively lipolytic, three enterotoxic strains of *Staph. aureus* were used as test organisms for this experiment. In the first group of experiments the basal part of the medium was prepared as for the complete medium, and distributed in 60.0 ml. quantities. The constituents to be added were dissolved in this and the volume made up to 100.0 ml. with distilled water. The medium was then divided into two portions to one of which fat suspension was added to a final concentration of 0.3 per cent, and tests were carried out in parallel. This was done to find out whether, in the absence of any specific amino-acid, fat might have a growth-promoting effect. Small volumes were withdrawn from each batch

of modified medium to test for growth in fluid medium, then aqueous agar was added. Plates were poured and dried thoroughly before inoculation. They were incubated at 37°C. for one week, and examined daily.

All these modified media except one, showed good growth and lipolysis as indicated by zones of clearing, after seventy-two hours of incubation. The exception was that lacking aneurin, and it seemed that aneurin might be an essential nutrient. Corroboration for this theory was provided by the evidence of Peters and Nelson (1951), that thiamine chloride (aneurin chloride), was essential for the production of lipase by Candida lipolytica when growing in synthetic medium. However, the essential role of aneurin in the experimental conditions obtaining was not subsequently established, although medium lacking only aneurin did not support growth of these Staphylococci well, and the clearing which appeared after five days of incubation was very slight.

Further alterations in the medium served to show that only cystine was an essential nutrient for the production of lipase by these strains, in the conditions of the experiment. Cystine was one of the amino-acids found by Surgelle (1937) to be essential for the production of enterotoxin by Staphylococci.

No evidence was found in any of these experiments that these strains of Staphylococci could

utilize fat to augment their growth when deprived of other nutrients.

In the second group of experiments modifications were made in the basal part of the medium, and the other separate constituents added as for the complete medium. The medium was again divided into two parts, fat suspension being added to one and aqueous agar to both.

Growth in all cases was slow, and in some cases poor, only very small colonies developing after incubation at 37°C. for five days. When the growth was poor, no evidence of lipase production was found.

Two conclusions were drawn from the results of this experiment:

- (i) that, with the exception of cystine, no single constituent of the medium was essential for lipase production, so long as the other constituents supplied were adequate for growth;
- (ii) that the presence of fat in modified synthetic medium had no enhancing effect on the growth of organisms investigated.

Summary of Part II.

Two general conclusions were drawn from the experiments done in the second part of the work:

- (i) that the ability to produce lipase is widely distributed throughout the staphylococci;
- (ii) that lipolysis occurs in most strains which are biochemically and metabolically active.

The conclusions drawn from the individual experiments were as follows:

- (i) that solid media are more sensitive than fluid ones for demonstrating lipolysis, particularly with weakly lipolytic organisms.
- (ii) that there is no relationship between pigment and lipase production.
- (iii) that lipase occurs more frequently in strains with a high degree of biochemical activity.
- (iv) that there is no specific relationship between enterotoxin and lipase production.
- (v) that no direct relationship exists between lecithinase and lipase production.
- (vi) that non-lipolytic strains may produce lipolytic mutants which can be increased in number by subculture in medium containing fat, while lipolytic strains may lose their activity after prolonged subculture through medium without fat.
- (vii) that fat has no enhancing effect on the growth of Staphylococci in diluted or deficient medium.
- (viii) that cystine is the only essential amino-acid for the production of Staphylococcal lipase.

Part III.

A STUDY OF THE OPTIMAL GROWTH TEMPERATURES OF
VARIOUS PARASITIC AND SAPROPHYTIC SPECIES, IN
RELATION TO LIPOLYTIC ACTIVITY

The results of the first parts of the experimental work carried out in the course of this investigation have shown that the production of diffusible lipase is relatively infrequent among parasitic bacteria, which have an optimal growth temperature around 37°C. It is, however, a property which is more common among micro-organisms which can grow as saprophytes, whether these be the true saprophytes found in air, soil and water, or the commensals and potentially pathogenic heterotrophs found growing on various commercial products. From published literature it would appear that lipolytic activity is largely confined to organisms growing at low temperatures, frequently those of the range used in refrigeration, approximately -5 to +10°C, (Vickery, 1936). These observations suggested that temperature might play an important role in the development of lipase by bacteria, and also raised the question of whether normally parasitic bacteria, if they could be cultivated at temperatures lower than the usual range, would produce lipase at these temperatures, although they did not do

so when growing at their optimal temperature of 37°C.

To investigate some of these possibilities, strains of Bacterium coli which had been previously examined (Part I, 3, 2), were retested at different temperatures. Strains of Pseudomonas pyocyanea isolated from routine pathological specimens, and saprophytes isolated from samples of soil, water and raw milk, were similarly tested. The latter included strains of bacterium, Pseudomonas, Achromobacter, Chromobacterium, Bacillus, Staphylococcus and Micrococcus, all of which had been found by earlier workers such as Kendall, Day and Walker (1914) and Jensen and Urbain (1936), to be lipolytic. The genus Proteus which was also shown by Kendall, Day and Walker (1914) to be lipolytic was not, however, included in this study, since the spreading properties of many strains would require adjustment of the agar content of the medium, and such adjustment would introduce a variable in the conditions of the experiment.

1) Experimental Technique.

Four incubators were used at temperatures of 37°C, 30°C., 22°C. and 8°C, the lowest of these being kept in the refrigeration room. Nutrient fat agar medium was used for all tests, no special medium for saprophytes being introduced. The plates were incubated for one week at 37 and 30°C., for three weeks at 22°C.,

and for four weeks at 8°C., to allow for slower growth at lower temperatures.

The degree of growth of different organisms at varying temperatures was estimated by measuring the diameter of well-separated colonies with dividers.

All organisms were identified as far as possible by colony formation and the usual biochemical tests.

Samples of soil were collected by the method suggested by Salle (1943), but in this case the suspensions were prepared in nutrient broth instead of water. The supernatant fluid was subcultured on to nutrient agar, and the plates and bottles of soil suspension incubated at 22°C. Colonies were then subcultured on to nutrient agar slopes which were incubated for three days at 22°C. Some strains were lost at this stage, as they did not survive subculture on nutrient medium.

Samples of natural water were taken by methods similar to those of Eyre (1930) and Taylor (1942). The organisms isolated from them were tested in the same way as those isolated from soil samples.

Samples of raw milk were kindly given by permission of the Director of the United Dairies Central Research Laboratory, London. Each sample was plated out on nutrient and blood agar, and the plates incubated

at 30° and 22°C. for twenty-four hours. Colonies were then subcultured to agar slopes. They were tested biochemically and for lipolytic activity as described above.

Two lipolytic strains of Staphylococcus aureus were used as positive controls.

2) Organisms Isolated from Routine Pathological Material.

i) Strains of Bacterium coli.

Fifty strains of Bact. coli were retested on nutrient fat agar, at the four temperature levels being studied. Growth was good at all levels, but progressively slower at the lower temperatures.

No evidence of lipase production was found, either at the optimal temperature of 37°C. or at any of the lower temperatures used.

ii) Strains of Pseudomonas pyocyanea.

Fifteen strains of Pseudomonas pyocyanea isolated from pleural fluids, sputa, urine, pus and the lungs of experimental rats, were tested for the production of diffusible lipase.

All strains showed an optimal growth temperature of 37°C. Most strains grew more slowly at the other temperature levels, three only very poorly at 8°C.

There was no evidence of lipolytic activity at any of the temperatures studied.

3) Organisms Isolated from Soil, Water and Milk.

i) Soil Bacteria.

Seven samples of soil were cultured, and from these the following thirty-five organisms were isolated:

<u>Pseudomonas pyocyanea</u>	7 strains
<u>Achromobacter</u>	2 strains.
<u>Bacillus subtilis</u> types	14 strains
<u>Bacillus mesentericus</u> types	1 strain
<u>Bacillus anthracoides</u> types	7 strains
<u>Staphylococcus</u>	1 strain
<u>Micrococcus</u>	2 strains
<u>Corynebacterium</u>	1 strain

All strains except one grew well at all temperature levels used, but showed optimal growth at 22°C. The exception was a strain of Achromobacter which failed to grow at 8°C.

Three of the thirty-five strains isolated showed lipolytic activity, one of which was the strain of Achromobacter which failed to grow at 8°C. This strain showed a comparable degree of lipolysis at 37°, 30° and 22°C. The other two lipolytic organisms were strains of Bacillus subtilis, which showed slight lipolytic activity at 30°C, that is, above the temperature optimal for growth, but not at the other temperatures of incubation:

ii) Water Bacteria

Sixty-eight organisms were isolated from twelve samples of water from rivers and ponds. These strains were as follows:

<u>Bacterium coli</u>	3 strains
<u>Bacterium lactis aerogenes</u>	2 strains
<u>Pseudomonas pyocyanea</u>	13 strains
Achromobacter species	6 strains
Chromobacterium species	9 strains
<u>Bacillus subtilis</u>	8 strains
<u>Bacillus anthracoides</u>	3 strains
<u>Staphylococcus albus</u>	4 strains
Micrococci	13 strains
Corynebacterium species	5 strains
Yeast	2 strains
	<u>68 strains</u>

All but six of the sixty-eight strains grew well at the four temperature levels used. The six exceptions failed to grow at 37°C. The optimal temperature in all cases was 22°C., but in most cases growth was equally good at 30°C.

Ten of the sixty-eight strains showed lipolytic activity at one or more of the temperatures of incubation, most frequently at the optimal temperature for growth. Two strains of Pseudomonas pyocyanea which showed lipolytic activity only at 37°C., had been isolated from a pond at the controlled temperature of 85°F. (29.5°C.). The observations made on these ten lipolytic strains are shown in Table X.

Table X.

Growth and Lipolytic Activity of Bacteria
Isolated from Water

Organism	Temperature at which growth took place	Temperature at which lipolysis was apparent
<i>Pseudomonas pyocyanea</i>	8°; 22°; 30°C.	22°C. only
<i>Pseudomonas pyocyanea</i>	All four	37°C. only
<i>Pseudomonas pyocyanea</i>	All four	37°C. only
<i>Achromobacter</i>	All four	30°C. only
<i>Chromobacterium prodigeosum</i>	All four	22°C. only
<i>Chromobacterium ochraceum</i>	All four	22°C. only
<i>Staphylococcus albus</i>	All four	22°, 30°, 37°C.
<i>Micrococcus</i>	All four	22°C. only
<i>Micrococcus</i>	All four	30°C only.
<i>Micrococcus</i>	All four	22°, 30°C.
<i>Staphylococcus aureus</i> controls	All four	22°, 30°, 37°C.

iii) Milk Bacteria.

Twenty-two samples of raw milk were examined and from these one hundred and eleven organisms were isolated. These were identified as in Table XI.

Table XI

Micro-Organisms Isolated from Samples of Raw Milk

Type of organism	Number isolated.
Bacterium coli types	14
Bacterium lactis aerogenes	11
Bacterium faecalis alkaligenes	1
Pseudomonas pyocyanea	15
Achromobacter	8
Chromobacterium	1
Lactobacillus	1
Bacillus subtilis	8
Staphylococcus	15
Streptococcus	10
Micrococcus	21
Sarcina	3
Corynebacterium	3

All strains grew equally well at 37° and 30°C., moderately well at 22°C., and more slowly at 8°C., Fifteen strains failed to grow at 8°C. The average optimal temperature for growth was found to be 30°C.

Twelve of the one hundred and eleven strains were found to be lipolytic. Some details of the lipolytic activity of these strains at different temperatures are

shown in Table XII.

Table XII
Growth and Lipolytic Activity of Bacteria
Isolated from Milk.

Organism	Temperature at which growth took place.	Temperature at which lipolysis occurred.
<i>Pseudomonas pyocyanea</i> , two strains	All four	8°C.
<i>Pseudomonas pyocyanea</i>	All four	22°C; 30°C.
<i>Pseudomonas pyocyanea</i>	All four	8°, 30°, 37°C.
<i>Achromobacter</i>	All four	22°, 30°, 37°C.
<i>Bacillus subtilis</i>	All four	30°C.
<i>Staphylococcus</i> (unpigmented)	22°, 30°, 37°C.	22°, 30°, 37°C.
<i>Staphylococcus</i> (unpigmented)	22°, 30°, 37°C.	30°C.
<i>Micrococcus</i> , two strains	All four	30°, 37°C.
<i>Micrococcus</i> , one strain	All four	30°C.
<i>Micrococcus</i> , one strain	All four	8°, 22°C.
<i>Staphylococcus aureus</i> controls	All four	22°, 30°, 37°C.

Summary of Part III.

The majority of the two hundred and eighty-four strains studied grew over the usual wide range of temperature.

Twenty-two of the strains (7.7 per cent) showed lipolytic activity at one or more of the temperatures of incubation, and in the majority of cases, the degree of lipolysis was comparable at all temperatures at which growth occurred. In strains which showed lipolytic activity at one temperature only, this temperature was nearly always found to be the optimal temperature for growth.

There was no evidence to show that organisms which are ordinarily non-lipolytic at their optimal growth temperature, were capable of producing lipase when cultivated at other temperatures.

Part IV.

THE DEMONSTRATION OF LIPASE IN CULTURE FILTRATES
OF STRAINS OF STAPHYLOCOCCI AND A STUDY OF ITS
IMMUNOLOGICAL ACTIVITY

Strains of Staph. aureus isolated from food-poisoning material, which had produced very marked lipolysis on nutrient fat agar (Part. II, 4), were investigated further for their ability to produce a filterable lipase when grown in suitable conditions.

1) Preparation of Culture Filtrates by a Method Used for the Production of Staphylococcal Toxins.

The method recommended by Mackie and McCartney (1948) for the preparation of staphylococcal toxin was used. Semi-solid agar (0.8%), was prepared by adding aqueous agar (6.0%), to nutrient broth which was then transferred either to glass bottles stoppered with cotton-wool, or to small conical flasks, in volumes of 25.0 ml. This volume was selected to give the maximum surface area to the culture. Each container was inoculated with 5.0 ml. of a twenty-four hour broth culture of the organism to be tested. The cultures were incubated at 37°C., in an atmosphere of 25.0 per cent carbon dioxide, for three days. A buffered peptone broth was prepared by adding peptone to the broth used in the semi-solid agar, to raise the concentration to

approximately 4.0 per cent, (usually 3.0 gm. of peptone were added for every 100.0. ml. of broth), and mixing this peptone broth with an equal volume of a one-fifteenth molar solution of potassium dihydrogen phosphate. The buffered peptone broth was added to each culture in the proportion of 5.0 ml. to 25.0 ml. semi-solid agar, and the cultures reincubated as before, for a further three days.

The cultures were harvested by filtering first through filter paper to remove the semi-solid agar, and then through Chamberland filter candles. Seitz filters were not used because of their tendency to adsorb some of the toxin (Topley and Wilson, 1946), although they were used without apparent loss of activity by Burnet (1930) and Parish and Clark (1942).

Several different media were used for the preparation of the cultures, and varying periods of incubation were tried out. These will be described in section 3 below.

2) Testing of Culture Filtrates for Lipase.

Three techniques were used to estimate the lipolytic activity of the filtrates; a test-tube method using semi-solid agar, the plate and cylinder method and the drop method.

. (1) The Test-tube Method.

This method utilised semi-solid agar, with

and without the addition of Nile Blue sulphate. The filtrate was layered over the surface of the semi-solid agar, and the tubes were incubated at 37°C. for periods up to ninety-six hours.

A positive result was indicated by the formation of a clear blue band between 1/8 and 1/4 inch below the surface of the medium, with a narrow clear band immediately below and above it. The results were optimal after forty-eight hours of incubation. When the tubes were kept at room temperature for a week or longer, diffusion continued and the blue band spread downwards, forming a series of separate rings. The reason for this phenomenon was unknown. When dye was omitted from the medium, clear bands replaced the blue ones, but as these were much less distinct, the dye was used in all subsequent tests. The controls were heavy living suspensions of a lipolytic strain of Staph. aureus and of the non-lipolytic Bact. coli D 433, and one of the filtrates heated to 100°C. for forty-five minutes.

This technique had one serious disadvantage due to the fact that some batches of the medium did not decolourise during incubation, but remained green. A sharp differentiation between the blue band characteristic of a positive result, and the remainder of the medium was therefore not obtained. Other batches were partly decolourised when first removed from the

incubator, but rapidly regained their green colour from the surface downwards, when left on the bench at room temperature for thirty minutes or longer. This reversible loss of the green colour of the medium was considered to be due to the formation of the colourless leuco-base of the dye, and subsequent re-oxidation to the coloured form by atmospheric oxygen.

Several variations in technique were introduced in an effort to establish a stable decolourised form of this medium.

Concentration of the semi-solid nutrient fat agar.

The concentration of agar in the semi-solid nutrient fat agar was varied over the range 0.2 to 2.0 per cent, and 2.0 ml. filtrate layered over 10.0 ml. medium. The optimum concentration was found to be 0.4 per cent agar.

Concentration of dye solution.

The usual aqueous solution (0.1%) Nile Blue sulphate was used, 1.0, 1.5 and 2.0 ml. being added to 100.0 ml. semi-solid nutrient fat agar. The optimum concentration was found to be the same as that used in all plates, that is, 1.5 ml. per 100.0 ml. medium.

Addition of Sodium thioglycollate to the medium.

Sodium thioglycollate was added to the

semi-solid nutrient fat agar to establish reducing conditions which, it was thought, might bring about greater decolourisation of the medium. Varying concentrations over the range 0.1 to 1.0 per cent were tested. All rendered the medium yellowish-green during incubation, except at the surface layer which remained green, but on removal from the incubator, the green colour reappeared rapidly from the surface downwards. The sodium thioglycollate seemed to have the effect of making the blue bands formed by lipolytic filtrates less clearly defined.

Addition of sodium taurocholate to the medium.

Sodium taurocholate was added to the medium (with or without dye), in view of the claims of Turner (1929) and Persky, Ravin, Jacob and Seligmann (1951) that it made medium cleared or rendered blue by lipase, transparent. Although such effects had not been observed in earlier experiments (part I, reproduction of Turner's work), sodium taurocholate was now added to the medium to give final concentrations of 2 and 5 per cent. It produced no enhancing effect, and was found to turn the medium very dark brown in colour.

From the results of these experiments it was concluded that sufficient decolourisation of nutrient fat agar containing dye, to show clear

contrasts between positive and negative results, could not be obtained by this layering technique, in which the medium had to be used in deep columns in test-tubes. The method was, therefore, abandoned, and attempts made to demonstrate lipolytic activity of culture filtrates using plates.

(ii) The Plate and Cylinder Method.

This method was based on that of Heatley (1944) for the assay of penicillin. Plates of nutrient agar containing dye, were prepared and dried for an hour. Sterile porcelain cylinders were heated in a bunsen flame and embedded in the agar. Five cylinders were placed on each plate, the central position being reserved for the control fluid. The filtrates were transferred to the cylinders by capillary pipettes. The plates were incubated at 37°C. for varying periods, and during incubation the fluid filtered out and diffused through the medium. The control was a filtrate of a known lipolytic organism, the activity of the filtrate having been destroyed by heating at 100°C. for forty-five minutes.

All filtrates from lipolytic strains produced blue zones round the cylinders after incubation for eighteen hours. As incubation proceeded up to ninety-six hours, the zones enlarged slightly, and concentric rings formed. On further incubation the zones shrank, and contracted gradually with ageing and deterioration of the medium.

The zones produced by all active filtrates showed a comparable pattern in their concentric rings, a ring on a plate corresponding to a band in the test-tube method (i) above. The central ring, or area immediately round the cylinder was deep blue and relatively opaque and sometimes showed precipitation resembling the deposition of fatty-acid crystals, after ninety-six hours of incubation. The ring immediately surrounding this area was clear and transparent, usually violet in colour but sometimes colourless. In some cases this ring divided into two, one of which was blue in colour. The third band was always the narrowest and was clear and colourless, or had a pale pink tint. Sometimes another poorly-defined, colourless band developed outside the pinkish one.

The size of the zones produced by different filtrates was compared by measuring the diameter of each concentric ring by means of dividers, the size and intensity of the zone being taken as an indication of the potency of the lipolytic factor in the filtrate, or of its diffusibility. Tests were set up in duplicate, two sets of measurements being obtained for each filtrate.

If Nile Blue sulphate was omitted from the medium, zones of clearing without colour formed but differentiation into rings was much less distinct.

The effects are shown in Plates IV and V and typical examples of measurements of the diameter of

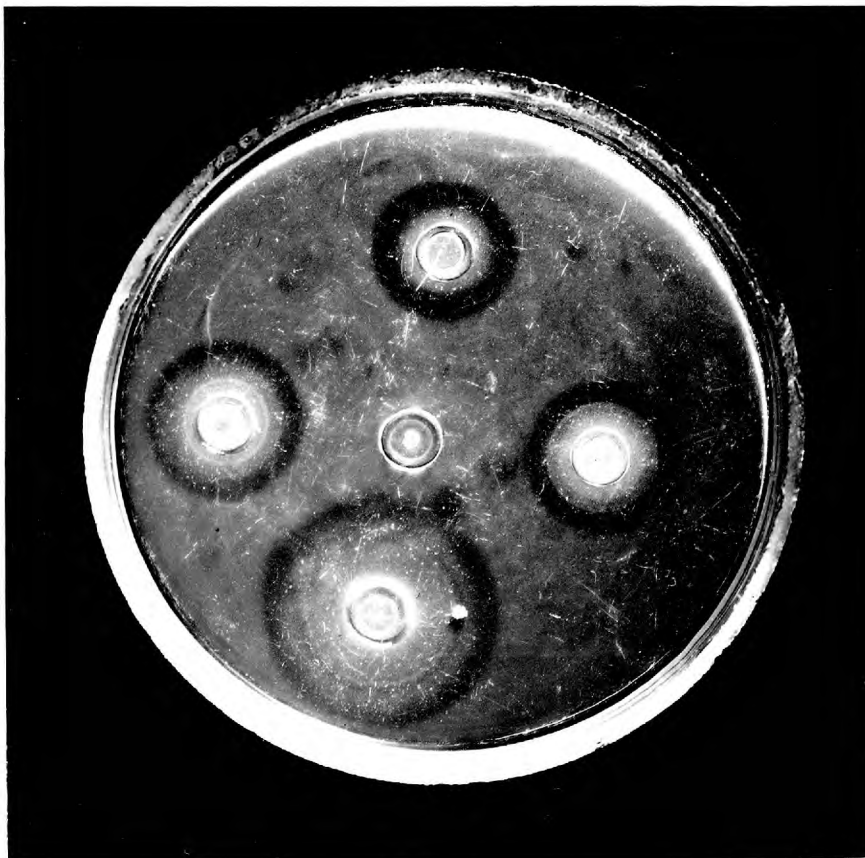


Plate IV

Zones of activity of filtrates from four lipolytic strains of Staph. aureus, on nutrient fat agar containing Nile Blue sulphate. The central cylinder contained a filtrate heated at 100°C. which produced no clearing in the medium.

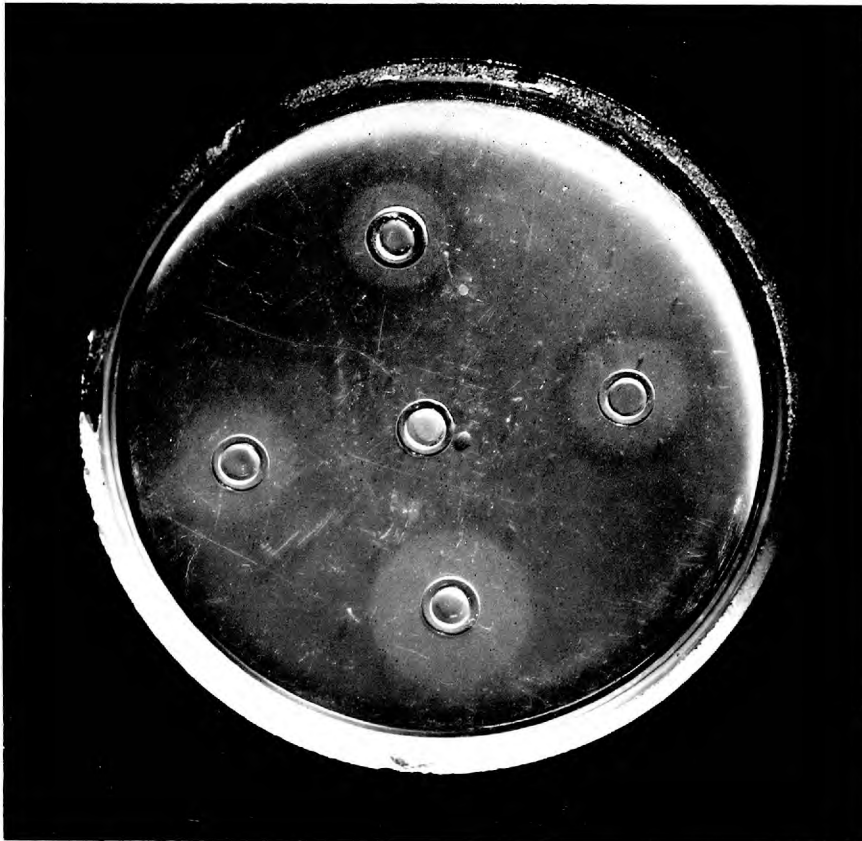


Plate V

Zones of activity of the same filtrates as shown in Plate IV, on nutrient fat agar without dye, when zone formation is much less distinct.

zones of activity are given in Table XIII.

Table XIII

The Lipolytic Activity of the Filtrate of a Culture of Staph. aureus W3 as Estimated by the Diameter of the Zone of Activity Measured at Varying Periods of Incubation.

Filtrate	Batch of Medium	Growth Time of culture	Colour Effect	Diameter of zone or band (mm.)			
				24 hrs.	48 hrs.	96 hrs.	120 hrs.
Staph. aureus W3	Digest Broth 3	6 days	Blue zone	11.0	11.0	11.5	11.5
			Violet band	13.5	14.0	13.5	14.5
			Pink band	-	18.0	19.0	20.0
Control	Digest Broth 3	6 days	nil	nil	nil	nil	nil

(iii) The Drop Method.

The third method used was one in which the cylinder was omitted from the plates, and drops of the filtrates were spotted directly on to the surface of the medium, from a pipette graduated to deliver fifty drops per ml. This method was used only on medium containing Nile Blue sulphate, and the plates were dried thoroughly before use. Positive results were shown by a blue zone in the medium where the drop of

filtrate had been absorbed. These zones were differentiated into concentric rings (Part IV, 2ii), similar to those observed when using the plate and cylinder technique.

Filtrates were titrated for lipolytic activity by diluting in normal saline over the range from neat to 1 in 5,000. The titre of a filtrate was taken as that dilution which just failed to show differentiation of the zone into concentric rings, after the plate had been incubated for forty-eight hours. This end-point was nearly always some four to eight times the lowest dilution showing demonstrable lipolysis. In lower dilutions there was little variation in the size of the zones, but as the dilution approached the end-point, the size and intensity of the zones tended to decrease. Repeated tests showed a variation of one two-fold dilution in some cases, so the average of triplicate results was taken as the titre.

The zones of activity formed by lipolytic filtrates tested by this method are shown in the mounted colour-film photomicrograph and in Plate X. Examples of the measurements of the diameter of zones from culture filtrates of two lipolytic strains of Staphylococci after diffusion for forty-eight hours, are given in Table XIIIa. The activity of two other strains of Staphylococci, in much higher dilutions, is shown in Plate VI.

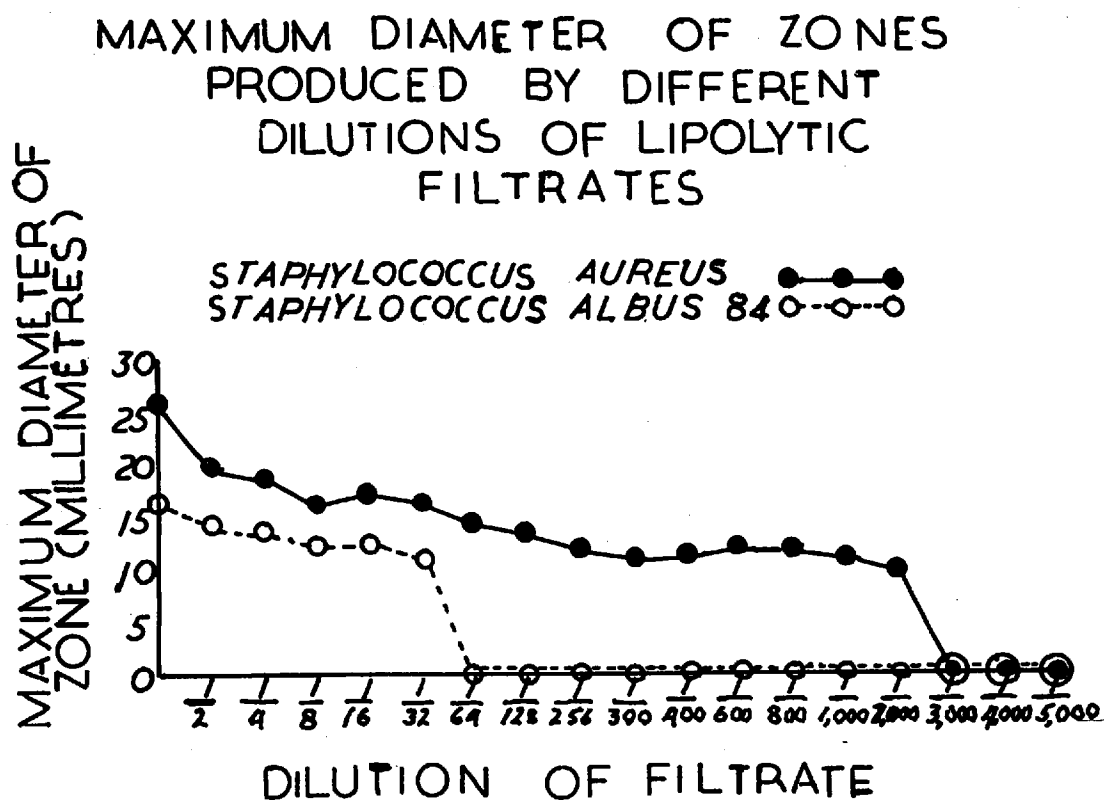


Plate VI.

The lipolytic activity of filtrates from a strain of Staph. aureus and albus in high dilutions.

Erratum: the change of scale in this table is due to the combination of the results of several experiments.

Table XIII(a)

Maximum Diameter (mm.) of Zones Formed by Dilutions of
Cultures Filtrates.

Filtrate	Dilution of filtrate.									
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
<u>Staph. aureus</u> M 3	20.0	20.5	20.0	20.0	19.5	16.0	15.0	13.0	12.0	-
<u>Staph. albus</u> 8	16.0	16.0	15.0	14.0	14.5	12.0	11.0	11.0	-	-

From Table XIV: Titre of Staph. aureus M-3 is 1/256

Titre of Staph. albus 8 is 1/128

The results showed that the plate and cylinder method of assay was more reliable than the semi-solid agar one. The drop method, however, gave the most satisfactory results, and was, therefore, used in subsequent experiments.

3) A study of the Optimal Conditions for the Production of Lipolytic Culture Filtrates.

Four experiments were done to determine the optimal conditions for producing lipolytic filtrates.

(i) Length of Growth Period.

The time of incubation of the cultures was increased from six to twelve and twenty days, the

buffered peptone broth being added to the semi-solid agar after six and ten days respectively. The zones of activity of three filtrates of Staph. aureus W 3 are shown in Plate VII and examples of measurements of the zone diameters are given in Table XIV.

Table XIV.

The Lipolytic Activity of Culture Filtrates of Staph. aureus W 3 grown for Different Periods of Time.

Filtrate	Growth time of culture	Medium	Colour effect	Diameter of zone (mm.).
1	6 days	Digest broth, batch 3	Blue zone Violet band Pink band	12.0 18.0 22.5
Control	6 days	Digest broth batch 3	Nil	Nil
2	12 days	Digest broth batch 3	Blue zone Violet band Pink band	11.5 23.5 24.5
Control	12 days	Digest broth batch 3	Nil	Nil
3	20 days	Digest broth batch 3	Blue zone Violet band Pink band	16.5 19.5 24.5
Control	20 days	Digest broth batch 3	Nil	Nil

The results of this experiment showed that no increase in lipase production could be brought about by growing the organisms for a longer period of time than that recommended for the production of staphylococcal toxins.

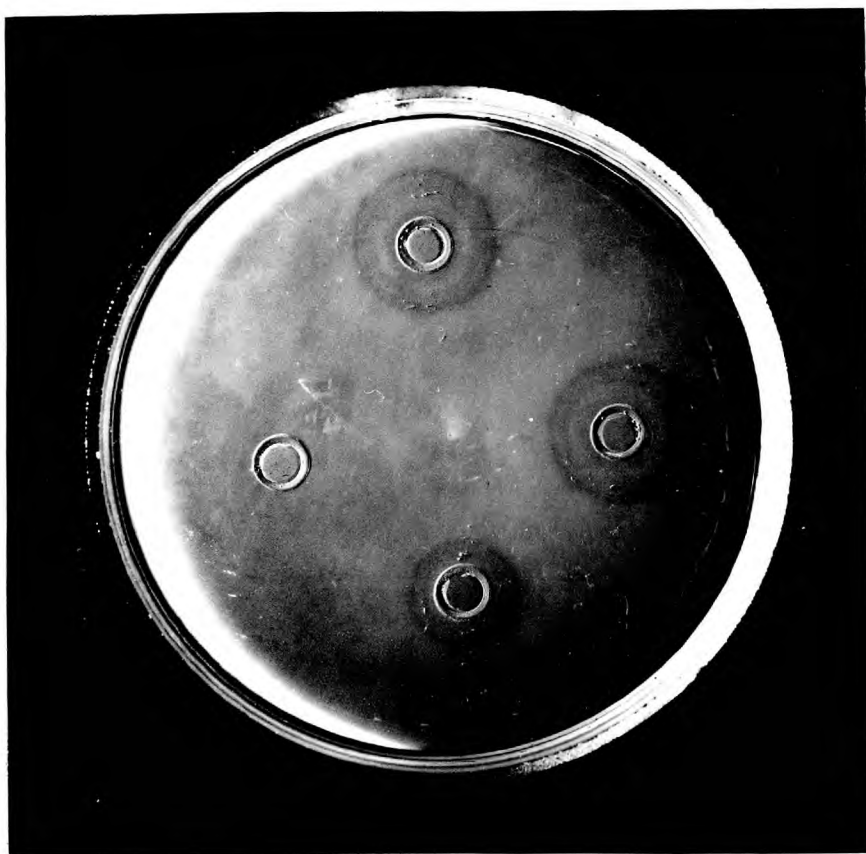


Plate VII

Zone of activity, showing concentric ring formation, produced by three culture filtrates of Staph. aureus W 3, grown for 6, 12 and 20 days respectively. The heated control filtrate shows no zone of activity.

The time of growth was then shortened to twenty-four and forty-eight hours to determine whether lipase would appear in culture filtrates after standing, as a result of conversion from its precursor if this were already present in the filtrate, as was observed by Elliott (1945; 1950) with streptococcal proteinase. Reducing conditions were established in the medium by adding sodium thioglycollate to give a final concentration of 0.1 per cent. The hydrogen ion concentration of the medium was varied by adding glucose in final concentrations of 0.5 and 1.0 per cent.

Three sets of cultures were prepared, two of which contained glucose. All were filtered without the addition of buffered peptone broth, and the pH and lipolytic activity of each was measured. Each filtrate was then divided into two parts, and sodium thioglycollate added to one. Each part was then divided again, and one half stored at 4°C. and the other at room temperature for one week. The filtrates were then retested for lipolytic activity.

Only one of three filtrates tested showed any lipolytic activity after growth for twenty-four and forty-eight hours, and that was very slight, the zones being small and poorly differentiated. After storage for one week the activity shown at twenty-four hours had been lost, while that showing after forty-eight hours was

retained but not increased. In no case was activity developed or increased as the result of storage. A typical example of the results from one of the filtrates is given in Table XV.

Table XV
Lipolytic Activity of Twenty-four and Forty-eight Hour
Culture Filtrates.

Filtrate	Time of culture	% of glucose added.	pH of filtrate	Diameter of zone of lipolytic activity (millimetres)				
				Immediate	After storage for one week.			
					4°C.	R.T.		
					Filtrate	Filtrate + sod. thio.	Filt-rate	Filtrate + sod. thio.
Staph. aureus M 3	24 hrs.	nil	8.0	11.5	nil	nil	nil	nil
		0.5	5.5	13.5	nil	nil	nil	nil
		1.0	5.0	nil	nil	nil	nil	nil
	48 hrs.	nil	8.0	13.5	15.0	14.0	13.0	15.0
				15.0	16.5	16.0		16.5
		0.5	7.5	14.0	15.5	17.0	nil	15.0
				15.0	18.0	18.0		18.0
		1.0	6.5	10.0	12.0	nil	nil	19.0
Control M 3	6 days	nil		17.0	19.0	18.0	19.0	19.0
				18.0	21.0	20.0	21.0	20.0
Control heated	6 days	nil		nil	nil	nil	nil	nil

The results did not indicate the free existence of the precursor of the lipase in the filtrate, as demonstrated by its spontaneous conversion to active lipase. If, however, the precursor was present in the filtrate, there was no evidence that its conversion to active lipase could

be stimulated either by the reducing conditions established by addition of sodium thioglycollate, or by an increase in the hydrogen ion concentration of the medium.

(ii) The Production of Filterable Lipase in Different Batches of Infusion and Digest Broth.

A strain of Staph. aureus W 3 was grown in five different batches of semi-solid agar for six days, in an atmosphere of 25 per cent carbon dioxide, at 37°C. The filtrates were assayed by the plate and cylinder technique, to determine which medium produced the most active filtrate. The inoculum for each culture was 5.0 ml. of a twenty-four hour culture grown in the same broth as that used to prepare the semi-solid agar.

The semi-solid agar was prepared from the following batches of broth:

- A) beef infusion broth, giving a final pH 7.6 after growth for 24 hours.
- B) beef digest broth giving a final pH 8.2 after growth for 24 hours.
- C) beef digest broth giving a final pH 8.0 after growth for 24 hours.
- D) beef digest broth giving a final pH 7.9 after growth for 24 hours.
- E) beef digest broth giving a final pH 7.0 after growth for 24 hours.

Batches of medium C, D and E were found to be satisfactory, growth being equally profuse in all. Batches A and B gave less potent filtrates. Examples of measurements of the zones of diffusion produced in

forty-eight hours, by filtrates of Staph. aureus W 3 grown in batches A, B and C, are shown in Table XVI. Plate VIII shows the zones of activity produced by these filtrates and by a heated control.

When the tests were repeated, varying the concentration of Nile Blue sulphate in the nutrient fat agar plates from 0.0015 to 0.004 per cent, similar results were obtained.

Batch C was the medium used in all subsequent experiments.

Table XVI.

Lipolytic Activity of Culture Filtrates of
Staph. aureus W 3

Grown in Different Batches of Medium.

<u>Filtrate.</u>	<u>Batch of medium.</u>	<u>Maximum diameter of zone.</u> (mm)
Staph. aureus W 3	A	12.0
	B	15.5
	C	21.0
	D	18.0
	E	20.0
Controls (heated)	A, B, C, D, E.	nil.

(iii) The Production of Lipolytic Filtrates in Large Volumes of Medium.

An attempt was made to increase the volume of filtrate produced in each culture. This was done by growing the organism in 500.0 ml. of semi-solid agar,

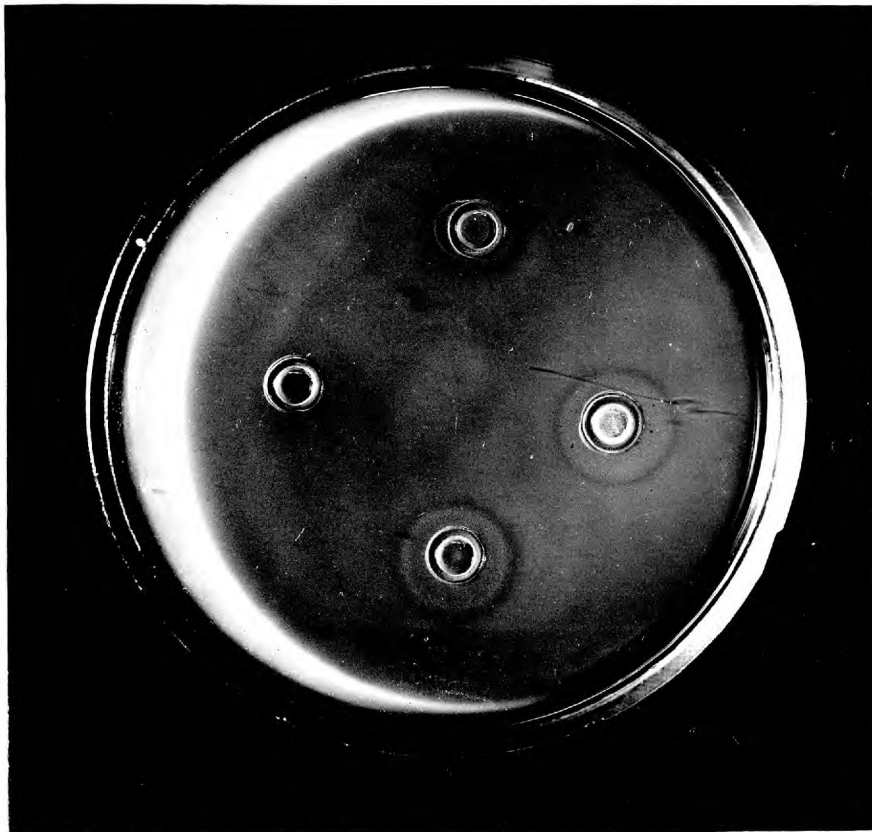


Plate VIII

The lipolytic activity of three filtrates from cultures of Staph. aureus W 3 grown in infusion and digest medium batches A, B and C, for six days.

in a conical flask (2-litre capacity), stoppered with a rubber bung through which a glass tube was fitted. The flask was inoculated and incubated in the usual way.

Repeated attempts showed that the filtrates of bulk cultures had only a small proportion of the activity which they had when grown in small volumes of medium. The zones of activity were faint in colour, poorly defined and undifferentiated. An example of the activity of one filtrate, estimated by measuring the diameter of the zones of activity is given below.

Filtrate of bulk culture: 6 days, diameter of zone of	
activity.....	12.5 mm.
12 days, " " " " "	13.0 mm.
Filtrate of 25 ml. culture: 6 days, diameter of zone of	
activity.....	22.0 mm.
12 days " " " " "	21.5 mm.

From these results it was apparent that satisfactory lipolytic filtrates could not be obtained by culture on a large scale.

(iv) The Effect of Growing Cultures in Absence of Carbon Dioxide on Lipase Production.

Investigation of the experimental conditions for the production of haemolysins by Staphylococci, was made by Burnet (1930), who showed that while carbon dioxide was essential during culture for aureus strains, albus strains and albus variants of aureus strains could produce haemolysins without the addition of carbon dioxide, provided that the reaction of the medium was on the acid side of neutrality.

The effect of these conditions on the production of lipase was investigated as follows. Digest broth and semi-solid agar were used simultaneously, the reaction of both being adjusted to pH 6.0 and pH 8.0. The medium was inoculated and incubated at 37°C. for six days, buffered peptone broth being added to the semi-solid agar after three days. The test organisms were Staph. aureus W 3 and M 3 and Staph. albus 39 and 84. The cultures were filtered through Chamberland candles and the filtrates tested for lipolytic activity.

Filtrates of Staph. aureus and albus both showed some lipolytic activity after culture without carbon dioxide. In both cases, however, the zones were smaller, paler in colour and poorly defined, compared with those formed when the culture was supplied with carbon dioxide. It was, therefore, concluded that, although carbon dioxide is not essential for the production of diffusible lipase by Staphylococci, it should be supplied during culture if the maximal activity is to be obtained.

An example of measurements of the diameter of the zones of activity of two of the strains studied, is given in Table XVII.

Table XVII

Maximum Diameter of the Zone of Lipolytic Activity of
Filtrates from Cultures Grown without Carbon Dioxide.

Organism	Medium	Reaction of medium (pH)	Diameter of zones (mm)
Staph. aureus W 3	Digest broth	6.0	12.0
		8.0	14.0
	Semi-solid agar	6.0	16.0
		8.0	13.0
Control	Semi-solid agar with carbon dioxide	7.8	24.0
Heated			nil
Staph. albus 39	Digest broth	6.0	14.5
		8.0	15.0
	Semi-solid agar	6.0	20.0
		8.0	14.0
Control	Semi-solid agar with carbon dioxide	7.8	20.5
Heated			nil

4) Some Physical Properties of Staphylococcal Lipase.

(i) Heat Resistance.

The heat stability of staphylococcal lipase was tested by heating the filtrates at different temperatures for varying periods of time.

Filtrates heated for short periods of time produced zones of lipolytic activity which differed little from those of unheated filtrates. Heating for longer periods of time resulted in partial inactivation and modification of the zones produced. The zones of lipolytic activity produced by heated filtrates were usually maximal after forty-eight hours of incubation, but occasionally very faint, undefined blue zones developed up to ninety-six hours of incubation. These were disregarded for comparative purposes.

The activity of culture filtrates of four strains of Staphylococci heated at 56°, 70°, 85° and 100°C. for varying periods of time, is shown graphically in Plate IX.

The results indicated that, compared with the majority of enzymes which are totally inactivated at 65°C. staphylococcal lipase is relatively heat stable, when in solution at or near pH 8.0. In this respect, it resembles crystalline trypsin which in acid solution is not wholly destroyed by heating at 100°C. (Sumner and Myrback, 1950).

EFFECT OF HEAT ON LIPOLYTIC ACTIVITY OF FILTRATES HELD AT DIFFERENT TEMPERATURES

STAPHYLOCOCCUS AUREUS ●—●—●
STAPHYLOCOCCUS ALBUS 39 ○-○-○

MAXIMUM DIAMETER OF ZONE (MILLIMETRES)

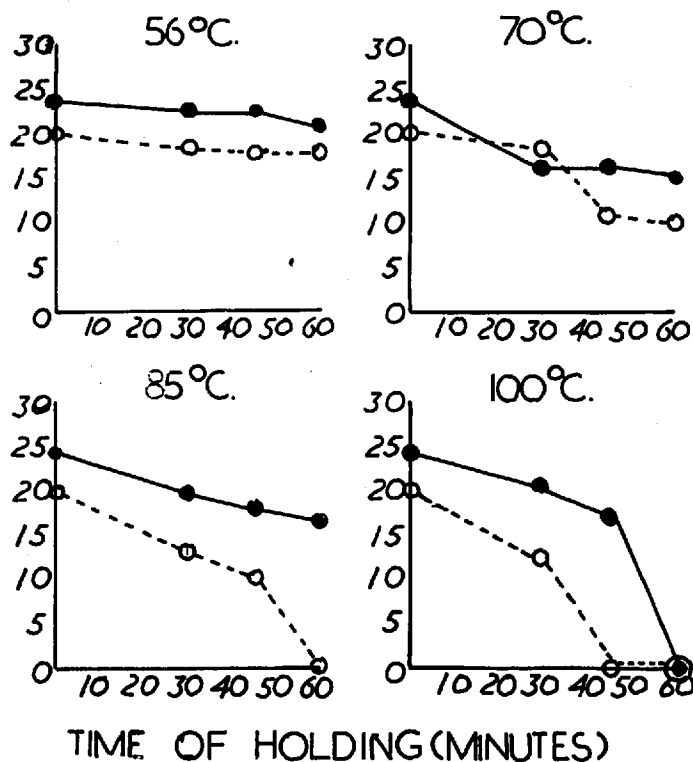


Plate IX

The effect of heat on lipolytic activity of staphylococcal culture filtrates.

(ii) Stability of Staphylococcal Lipase on Storage.

The period of time for which a lipolytic culture filtrate retained its activity was measured by storing the filtrate at 4°C. and testing at intervals of several weeks. The tests were made using the plate and cylinder method of assay, and continued over nine months.

Nineteen filtrates from three strains of Staph. aureus and five strains of Staph. albus, from cultures grown in different batches of medium, were studied. Those from one strain of Staph. aureus and two strains of Staph. albus lost all their lipolytic activity during the first month of storage, while those from another strain of Staph. aureus lost their activity after storage for four months. The remaining eleven filtrates showed only a very gradual loss of lipolytic activity, which was apparent more by loss of differentiation of the zones produced than by decrease in their size.

Examples of the measurements of the zones produced by the filtrates which retained their lipolytic activity largely undiminished through nine months of storage, are shown in Table XVIII.

Table XVIII

Diameter of Zones of Lipolytic Activity of Culture Filtrates
of Staphylococci during Storage at 4°C. (mm.)

Culture Filtrate	Time of storage at 4°C. (days)					
	0	30	96	150	225	275
Staph. aureus W 3	23.0	24.0	16.0	15.0	15.0	14.5
Staph. aureus C 25	18.0	21.0	20.0	18.5	17.0	16.0
Staph. albus 8	20.0	18.0	18.0	16.0	16.0	16.0
Staph. albus 84	17.5	18.0	17.5	17.0	16.5	17.5

(iii) The Lipolytic Activity of Fractions Isolated From
Staphylococcal Filtrates by Means of Precipitation
with Ammonium Sulphate and Paper Electrophoresis.

Preliminary experiments were carried out in an attempt to achieve separation and some purification of the lipase from the crude filtrate by precipitation with ammonium sulphate (80.0%) and by paper electrophoresis, using techniques recently developed by Strain (1939), Consden, Gordon and Martin (1941), Dent (1946), Flynn and de Mayo (1951) and Sophian and Conolly (1952).

Precipitation of the Protein.

Culture filtrates of lipolytic, enterotoxigenic strains of Staph. aureus were prepared as described in

Part IV, 1, and mixed with four volumes of saturated ammonium sulphate. The precipitate which formed was separated by centrifuging, and washed in saturated ammonium sulphate solution. The washed precipitate was dissolved in water, and the solution tested for lipolytic activity by the plate and cylinder technique, using a control for the ammonium sulphate. The ammonium sulphate was then removed from the solution by dialysis against distilled water. Forty-eight hours were necessary for the complete removal of the ammonium sulphate as estimated by the use of Nessler's Reagent. The dialysate was then tested for lipolytic activity and found to be almost as active as the whole filtrate. It was then evaporated to dryness over phosphorous pentoxide in a dessiccator. The precipitate was weighed and the yield found to be 170.0 mgm. from 1,000 ml. filtrate. Nearly all the precipitate (90.0%) redissolved in 3 ml. distilled water.

The Electrophoretic Apparatus.

The apparatus used consisted of a shallow perspex tray with two troughs to hold the buffer solution, sodium barbiturate pH 8.6. The paper used was Whatman's filter paper, No. 1 and 31 (extra thick). The thinner paper proved easier to handle and stain, but the thicker one macerated more easily and therefore elution of the adsorbed material was more complete. Narrow strips of the paper were stretched over perspex rollers between the carbon electrodes with the ends

dipping into the buffer solution. The electric current was applied from a dry battery, and the potential applied was 120 volts.

Electrophoresis

The dried precipitate was dissolved in the minimum volume of distilled water (3.0 ml.) and a known volume (0.1 ml.) was distributed evenly from a pipette along a pencilled line drawn across the paper strips, about six centimetres from the cathode end. The tray was covered with a glass plate and left to stand until the buffer solution had saturated the paper strips. The potential was then applied, and maintained for periods varying from six to sixty hours in different experiments.

The Distribution of Lipase on the Paper Strips.

(a) As Shown by the Elution Method.

Each paper strip was divided into two parts, and one part was cut into twelve pieces, the first of which was on the cathode side of the pencilled starting line. Each of these twelve pieces was put into 1 ml. distilled water in a test-tube, macerated with a glass rod and left at room-temperature for sixteen hours. The supernatants were then pipetted off and tested for lipolytic activity by the plate and cylinder technique. Some examples of the measurements made are given in Table XIX.

(b) As Shown by Staining Methods.

The other half of the paper strip was treated by

various staining methods. These included the use of the ninhydrin action (Abderhalden and Schmidt, 1913) as modified by Consden and Martin (1944); Gomori's method for the detection of lipase in tissue sections (Gomori, 1944); and the use of alpha-naphthyl acetate and the diazo reaction (Seligman, 1948). This latter method was the only one which gave moderately successful results, all the others being completely unsatisfactory.

This method was modified from that of Nachlas and Seligman (1948) for the histochemical demonstration of esterase. The substrate was prepared by dissolving 50.0 mgm. of alpha-naphthyl acetate in 0.5 ml. acetone, and making the solution up to 50.0 ml. with sodium phosphate buffer solution, pH 7.4, then adding 50.0 mgm. of ortho-dianisidine. The solution was filtered and immediately thereafter the strips were immersed in it for three minutes. During this time, the alpha-naphthol liberated by the lipase from the substrate, coupled with the diazonium compound and an insoluble purplish-red pigment appeared on the paper. When the strips were washed in distilled water and dried, a dark purplish-red area, approximately 8.5 cm. in length, extended along the paper. No coloured area appeared if the dialysate was boiled for forty-five minutes before electrophoresis.

When the strip of paper was divided, and the two halves examined for lipolytic activity by different methods, the coloured area developed on one half

corresponded to that on the other half showing lipolytic activity by the elution method.

Two other compounds, beta-naphthyl acetate and alpha-naphthyl laurate, were substituted for alpha-naphthyl acetate, but neither of these substances gave a colour reaction.

The alpha-naphthyl acetate and alpha-naphthyl laurate were kindly given by Dr. A.G.E. Pearse, and the beta-naphthyl acetate by Dr. J.C.S. Paterson of the Postgraduate Medical School of London.

Table XIX

Lipolytic Activity of Eluates of Precipitated Protein Material from Staphylococcal Culture Filtrates, After Paper Electrophoresis.

Position on Paper strip	Intensity of colour of zone	Diameter of zone of activity after 48 hours. (mm.)			
		Strip 1 (potential applied 6 hours)	Strip 2 (potential applied 18 hours)	Strip 3 (potential applied 32 hours)	Strip 4 (potential applied 60 hours)
- 1	nil	nil	nil	nil	nil
+ 1	Very deep blue	11.0	13.5	11.0	13.5
2	Intense blue	11.0	13.0	12.0	12.0
3	Less intense blue	10.5	12.0	12.0	11.0
4	Paler blue	10.0	11.0	11.5	nil
5	Faded blue	nil	10.5	10.5	nil
6	nil	nil	nil	nil	nil
7	nil	nil	nil	nil	nil
8	nil	nil	nil	nil	nil
9	nil	nil	nil	nil	nil
10	nil	nil	nil	nil	nil
11	nil	nil	nil	nil	nil
12	nil	nil	nil	nil	nil
Controls: Precipitate undiluted	Very deepblue	16.0	17.5	17.5	16.5
heated at 100 C.	nil	nil	nil	nil	nil

The protein fraction was found to travel fairly rapidly and to spread half-way along the paper strips, as estimated by both the elution and the staining method.

The experiments were not, however, proceeded with further, for two reasons. Firstly, the staining of the material on the paper strips proved technically very difficult. Secondly, at that time, no quantitative method for estimating lipase such as that used later during the production of anti-lipolytic antibodies, had been developed. Although the plate and cylinder method of assay, developed for undiluted filtrates, was used for precipitates and eluates also, it was realised that this method would inevitably give only a rough approximation of the relative potency of different fractions of the filtrates.

Part V:

THE PRODUCTION OF ANTI-LIPOLYTIC ANTIBODIES IN
RABBITS AGAINST STAPHYLOCOCCAL LIPASE, AND A
STUDY OF THE ANTIGENIC SPECIFICITY OF THE LIPASE

No references have been found in published literature to the production of anti-lipase in animal sera, as the result of inoculation with bacterial lipase. Nitzescu and Benetato (1933), however, claim to have brought about a decrease in the lipolytic activity of the serum of rabbits by the intravenous injection of three increasing doses of an extract of swine pancreas. They suggested that this decrease might be due to the formation of an anti-lipase.

In the present work attempts have been made to produce anti-lipase in rabbits, firstly by immunisation with living cultures of avirulent lipolytic strains of Staph. albus and secondly by inoculation with filtrates of these strains and of lipolytic strains of Staph. aureus.

*

* 1) The Production of Anti-Lipolytic Antibody in Rabbit Serum by Immunisation with living Cultures of Staph. Albus

No difference was observed between the antisera prepared by injection of living cultures of albus strains and those prepared from the culture filtrates of these strains.

Before inoculation samples of serum were taken from each rabbit and set aside for examination for naturally occurring anti-lipolytic activity.

Cultures were grown for twenty-four hours in digest broth, then diluted 1 in 10 and inoculated intravenously into rabbits in a dosage of 0.1, 0.2, 0.3

and 0.4 ml., on successive days, followed by four days of rest. This procedure was repeated the following week and in two subsequent weeks, when undiluted cultures were used. Samples of blood were taken four days after the last immunising dose. Three rabbits were used for each culture.

2) The Production of Anti-lipolytic Antibody in Rabbit Serum by Immunisation with Culture Filtrates of Staph. aureus and albus.

Filtrates were given on three successive days in a dosage of 0.1. 0.2 and 0.5 ml. followed by four days of rest. This procedure was repeated the following week, and in two succeeding weeks when the dosage was increased to 0.5, 0.75 and 1.0 ml. Filtrates of aureus strains proved too toxic to be used without treatment with formalin. This was, therefore, done using the method of Dolman and Kitching (1935), for the production of staphylococcal toxoid. Formalin was added to the filtrate to give a final concentration of 0.15 per cent and the filtrate incubated at 37°C. Samples were tested at intervals for their haemolytic activity against rabbit erythrocytes, and the bulk filtrate kept in the incubator until the undiluted sample caused no haemolysis of a 0.1 per cent suspension of rabbit erythrocytes in one hour at 37°C. Filtrates were inactivated in this way after a period varying from one to three weeks.

Rabbits were then injected intravenously with the formolised filtrates, in the graded doses, and by the method given above. The series of injections was given four times, with intervals of about two weeks between, before the sera were used in the test to be described.

3) Titration of Culture Filtrates for Lipase.

Filtrates were titrated for lipolytic activity by making doubling dilutions in normal saline, up to 1 in 512, and delivering drops by means of a 50 ml. dropping pipette, on to the surface of nutrient fat agar containing Nile Blue sulphate (0.002%), which had been overdried for one hour (as described in Part IV, 2, iii). The plates were incubated in the usual position at 37°C. for forty-eight hours. A positive result was shown by a blue zone in the medium where the drop of filtrate had been absorbed. These zones were differentiated into concentric rings. (Part IV, 2, iii). In lower dilutions there was little variation in the size of the zones, but as the dilution approached the end-point, the size and intensity of the zones tended to decrease. An arbitrary degree of lipolysis was chosen empirically as the titre of the filtrate and was that dilution which just failed to show differentiation into concentric rings. This was some four to eight times the lowest dilution showing demonstrable lipolysis on the plate. The titre of the filtrate shown in the upper left hand quadrant of Plate X (to be described in section 4 below) is 1 in 128. Triplicate tests showed a variation

of one two-fold dilution in some cases, so the average of triplicate results was taken as the titre.

4) Titration of Immune Rabbit Sera for Antilipolytic Activity.

Sera were titrated for anti-lipolytic activity as follows: serial dilutions of the homologous filtrate were prepared and delivered on to nutrient fat agar plates as above (3). Drops of undiluted serum, and of serum diluted from 1 in 5 to 1 in 40, were mixed thoroughly with the drops of filtrate before these had had time to absorb, and after absorption, the plates were incubated as before. Duplicate plates were used in all titrations for anti-lipolytic antibody. Two controls were used; the first was dilutions of the filtrate alone, the second, dilutions of the filtrate mixed with undiluted normal rabbit serum. Typical results are shown in Plate X. The control plate (upper left hand quadrant) and the plate with drops of a mixture of the filtrate and undiluted normal rabbit serum (upper right-hand quadrant), show identical results. No evidence of any naturally-occurring anti-lipolytic antibody in rabbit serum was found. Undiluted immune serum mixed with drops of the filtrate (lower left hand quadrant), caused partial neutralisation of the filtrate undiluted and diluted 1 in 2, and complete neutralisation in all higher dilutions. When the immune serum was diluted 1 in 10, it neutralised the lipolytic activity of the filtrate only in dilutions higher than 1 in 16. With further dilution the anti-lipolytic activity of the immune serum decreased rapidly,

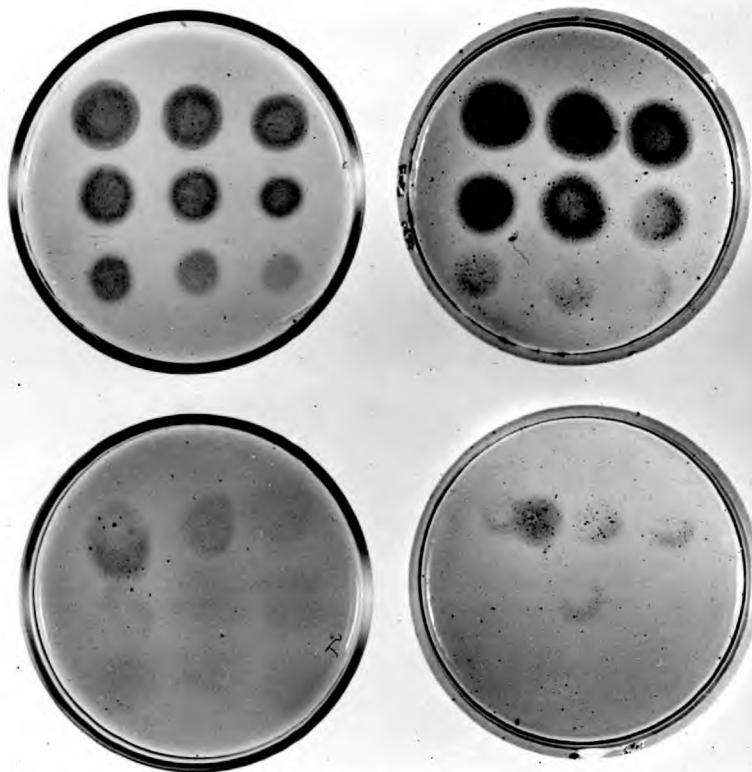


Plate X.

The lipolytic activity of a culture filtrate of Staph. aureus diluted serially up to 1 in 512, mixed with normal rabbit serum and with immune rabbit serum, undiluted and diluted 1 in 10.

until no appreciable effect was observed at 1 in 40. The effects described in Plate X can be more clearly seen in a colour photomicrograph, which for technical reasons cannot be included in this thesis.

Other examples of the anti-lipolytic activity of rabbit serum against the homologous filtrate, following the third series of injections in the course of immunisation described above, are shown in Table XX. It will be seen from these results that neutralisation is not always as complete as that shown in Plate X. Sera withdrawn during the course of immunisation did not neutralise the lipolytic activity of the filtrate completely when used undiluted, and in all dilutions from 1 in 5 to 1 in 40, showed only moderate or slight anti-lipolytic activity.

After a fourth series of injections undiluted immune rabbit serum was found to neutralise the lipolytic activity of some filtrates completely. There was no evidence of any pro-zone of inhibition of lipolytic activity with low dilutions of the anti-serum, such as has been observed in other anti-enzyme reactions, for example, the destruction of non-specific inhibitors in normal ferret sera by filtrates of Vibrio cholerae (Isaacs and Bozzo, 1951). The anti-sera were therefore used undiluted in subsequent tests.

Table XX.

Anti-lipolytic Activity of Normal and Immune Rabbit Serum
after Three Series of Injections Against the Homologous
Lipolytic Filtrates.

Filtrate	Anti-serum	Dilution of Anti- serum	Dilution of Filtrate.									
			0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Staph. aureus W 3	0	-	+++	+++	+++	+++	+++	++	++	+	+	trace
	Normal control	0	+++	+++	+++	+++	++	++	++	+	+	"
	Immune W 3	0	+	+	trace	-	-	-	-	-	-	-
		1/5	++	+	+	trace	trace	-	-	-	-	-
		1/10	+++	++	+	+	+	trace	-	-	-	-
		1/20	+++	+++	++	++	+	+	trace	trace	-	-
		1/40	+++	+++	++	++	+	+	+	"	trace	-
Staph. aureus M 3	0	-	+++	++	++	+	+	+	+	"	"	-
	Normal control	0	+++	++	++	+	+	+	+	"	-	-
	Immune M 3	0	+	+	+	trace	-	-	-	-	-	-
		1/5	++	+	+	+	trace	-	-	-	-	-
		1/10	+++	+++	++	++	++	+	+	trace	-	-
		1/20	+++	+++	+++	++	++	trace	trace	"	-	-
		1/40	+++	+++	+++	++	+	+	"	"	-	-
Staph. albus 8	0	-	+++	+++	++	++	++	+	+	+	trace	-
	Normal control	0	+++	+++	++	++	++	+	+	+	"	-
	Immune 8	0	+	+	trace	-	-	-	-	-	-	-
		1/5	++	+	+	trace	trace	trace	-	-	-	-
		1/10	+++	++	+	+	+	"	trace	-	-	-
		1/20	+++	++	++	+	+	+	+	+	-	-
		1/40	+++	++	++	+	+	+	+	+	-	-
Staph. albus 39	0	-	+++	++	++	+	+	+	trace	-	-	-
	Normal Control	0	+++	++	++	+	+	+	"	-	-	-
	Immune 39	0	+	+	trace	trace	-	-	-	-	-	-
		1/5	+	+	+	+	trace	trace	-	-	-	-
		1/10	+++	+++	++	+	+	"	-	-	-	-
		1/20	+++	++	++	++	+	+	trace	-	-	-
		1/40	+++	++	++	+	+	+	"	-	-	-

5) The Antigenic Specificity of Staphylococcal Lipase.

Thirty filtrates were prepared from strains of Staphylococci, sixteen from strains of Staph. aureus and fourteen from Staph. albus. The lipolytic activity of the filtrates was assayed by the drop method as described above. The most potent filtrate had a titre of 1 in 512. All those which had a titre of less than 1 in 32 were discarded. Two filtrates from strains of Staph. aureus and two from Staph. albus were omitted from the main test for this reason. Eight other filtrates from strains of Staph. albus were also omitted because they showed no lipolytic activity, although the organisms themselves were fairly active on nutrient fat agar. Absence of activity in these filtrates may have been partly due to adsorption of the lipase during filtration. It is probable, however, that it was mainly due to the lability of the enzyme produced by some strains, since the activity of some, but not all, the filtrates of albus strains showed rapid deterioration on storage (Part IV, 4, ii).

Five anti-sera were prepared, two against strains of Staph. albus using the living organism and the culture filtrate, and three against strains of Staph. aureus, using formolised filtrates only. These were tested against the eighteen filtrates, fourteen of which were from strains of Staph. aureus and four from Staph. albus.

Sodium thioglycollate was again added to the medium as in earlier experiments (Part IV, 2, i), but was not found

to have any enhancing effect on the action of the lipase in the filtrates such as it was observed by Elliott (1945, 1950) to have on streptococcal proteinase.

A summarised record of the results of these experiments, showing only those obtained with undiluted anti-sera, after the completed course of immunisation, is given in Table XXI.

Table XXI

Anti-lipolytic Activity of Five Anti-sera against Eighteen Culture Filtrates of Lipolytic Strains of Staph. aureus and albus

Anti-serum	Titre of filtrate after mixing with undiluted anti-serum																		
	From <u>aureus</u> strains														From <u>albus</u> strains				
	W 1	W 3	W 4	W 7	W 9	W 10	W 11	W 13	W 15	W 18	1	14	17	8	16	20	39		
Control	$\frac{1}{256}$	$\frac{1}{256}$	$\frac{1}{128}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{128}$	$\frac{1}{64}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{64}$		
W 3	-	U	-	U	U	-	-	U	-	-	-	-	-	U	$\frac{1}{4}$	-	-		
W 4	-	$\frac{1}{2}$	-	-	U	-	U	-	U	U	U	-	-	-	$\frac{1}{2}$	$\frac{1}{2}$	U		
M 3	$\frac{1}{4}$	$\frac{1}{2}$	-	U	$\frac{1}{2}$	$\frac{1}{2}$	U	-	-	-	-	U	-	U	U	U	-		
8	U	$\frac{1}{2}$	-	-	U	$\frac{1}{2}$	-	-	$\frac{1}{2}$	U	-	-	-	U	-	U	$\frac{1}{4}$	$\frac{1}{2}$	
39	$\frac{1}{4}$	$\frac{1}{8}$	-	-	U	U	-	U	$\frac{1}{4}$	U	-	-	U	U	-	$\frac{1}{2}$	U	-	

U = undiluted filtrate.

These results showed that, in some cases undiluted rabbit anti-serum neutralised the lipolytic activity of the homologous filtrate completely, the titre of filtrate W 4, for example, being lowered from 1 in 128 to nil. In other cases, however, the lipolytic activity was not completely neutralised, the titre of filtrate W 3 for example, being reduced from 1 in 256 to 1.

When the anti-sera were tested against heterologous filtrates, similar results were obtained. The titre of filtrate W 4, for example, was lowered from 1 in 128 to nil by all heterologous anti-sera, while that of filtrate W 3 was lowered from 1 in 256 to 1 in 2 by three heterologous anti-sera and to 1 in 8 by the fourth.

The results of the cross-neutralisation tests, therefore, suggested that staphylococcal lipases are not highly specific and showed those of the relatively small number of strains studied to be anti-genically homogeneous.

Discussion.

The first stage of this work was the establishment of a satisfactory medium for the rapid demonstration of a diffusible lipase produced by bacteria during the first forty-eight hours of growth in culture medium. In published literature references were found to approximately twelve different methods for demonstrating lipase production by bacteria, and these have been described in the section entitled Bacterial Lipolysis in vitro. Only two of these methods demonstrated lipase production during the phase of active growth of the organism. One was the titration method of estimating the free fatty-acids liberated, favoured by Trussell and Weed (1937), while the other utilised solid medium in which the fatty substrate was incorporated with or without an indicator. This latter method was the one favoured by Turner (1929), Collins and Hammer (1934) and Jensen and Grettie (1937). A reproduction of Turner's medium did not prove satisfactory and various other media were developed and tested. A modification of Turner's medium, in which the suspension of fat was prepared with the help of a mechanical emulsifier in place of a gum base, and in which the quantity of Nile Blue sulphate dye was reduced, was finally selected as fulfilling all the requirements of a rich and sensitive medium. Results on this medium were compared with those obtained by the

titration method in fluid medium, and it was found that agreement was good for actively lipolytic strains. Agreement was not so good with strains which were only feebly lipolytic, and for these solid medium was found to be the more sensitive. Nutrient fat agar, with or without dye, (Part I, 2), was, therefore, used as the standard medium for demonstrating the production of diffusible lipase.

A preliminary investigation of a small number of strains of seventeen genera, indicated that the Staphylococci were the chief producers of diffusible lipase. This observation was confirmed in the systematic investigation carried out (Part I, 3), which showed that among potentially pathogenic bacteria, the only genus in which the ability to produce diffusible lipase is widespread is the Staphylococci. In the other genera of pathogenic importance in which it was demonstrated, namely Pseudomonas and Clostridium, it was encountered only in a small number of strains. In view of this result the later parts of the work were confined to the genus Staphylococcus.

Lipase Production in Relation to Pigment Production and General Biochemical Activity.

A comparison of the percentage of strains producing lipolysis, encountered among aureus strains (75.0%), with those among albus strains of Staphylococci having a similar degree of general biochemical activity,

including the production of coagulase, and which might be regarded as albus variants (67.0%), showed that lipolysis was not related specifically to pigment production.

A comparison of the percentage of lipolytic strains in the latter group with that of other albus strains with moderate fermenting ability (17.0%), and with weak fermenting ability, suggested that lipolysis runs parallel to general biochemical activity, and is but one manifestation of it.

Lipase Production in Strains of Staphylococci Associated With Food Poisoning.

A collection of strains of Staphylococci isolated from material associated with outbreaks of food-poisoning, were examined for lipolytic activity. It was assumed, but not proven, that the majority, if not all of these strains produced enterotoxin, and it was hoped to determine whether any relationship existed between lipase and enterotoxin production.

Twenty-five of these strains were tested for the production of diffusible lipase, and for their ability to ferment carbohydrates and to produce coagulase and haemolysin. Strains of Staphylococci of comparable biochemical activity isolated from other pathological material and from innocuous foodstuffs, were used as controls.

All the strains associated with food-poisoning were found to be very actively lipolytic, while only approximately fifty per cent of those from innocuous foodstuffs were lipolytic. But, as nearly all the control strains from other pathological material were lipolytic also, no deductions could be made as to any specific relationship between lipase and enterotoxin production.

The Relationship Between Lipase Production and Haemolytic Activity on Blood-agar Plates:- The Production of Haemolysis by Lipase.

The possibility that lipase might be concerned in the production of haemolysis on blood-agar plates by Staphylococci, by breaking down the lipids contained in the cell envelope, was investigated in the experiments described in Part II, 5.

Seventy-two lipolytic strains of Staphylococci were grown on blood-agar and on nutrient fat agar. A considerable proportion, (19 out of 72), were found to be non-haemolytic. This suggested that lipase does not produce lysis of red blood cells in normal circumstances.

In subsequent experiments the same strains were grown on ordinary blood-agar and on blood-agar containing fat. Two out of fifty-three strains produced beta-haemolysis on fat-containing plates, but not on ordinary blood-agar. This observation suggested that when fat is present, some lipolytic strains which are ordinarily non-

haemolytic, may produce a haemolytic zone, probably due to lysis of the red blood cells by the fatty-acids liberated by the lipase. This zone of haemolysis did not appear to differ in any way from that produced on fat-free blood-agar.

The Occurrence of Mutants in Lipolytic and Non-Lipolytic Strains of Staphylococci.

The presence of mutants in lipolytic and non-lipolytic strains was investigated by plating out and studying individual colonies for lipolytic activity. (Part II, 6).

Two lipolytic strains did not produce any non-lipolytic colonies among 30,000 studied, while two non-lipolytic strains produced lipolytic mutant colonies in the proportion of 1 in 11,500 and 1 in 13,000 colonies respectively. This suggested that naturally occurring lipolytic strains may possibly be more stable than non-lipolytic ones.

The possibility of producing or increasing the proportion of mutants by a process of selection brought about by repeated subculture in conditions favourable to their development, was studied.

A lipolytic strain was subcultured repeatedly over fifty-five transfers in fat-free medium, and the resulting strain was completely non-lipolytic. This would not, however, appear to have been produced by a

process of selection of non-lipolytic variants from the original culture since the loss of lipolytic activity occurred uniformly in all colonies examined during the process of subculture. Moreover, the loss appeared to be permanent, since no lipolytic variant colonies were encountered from this strain after seventy-two further subcultures in medium containing fat. This artificially induced non-lipolytic strain, therefore, appeared to be more stable than the naturally occurring one investigated above.

Three originally non-lipolytic strains were subcultured in fat-containing medium. Two of these were those found to produce rare lipolytic mutants, described above. In one of these the proportion of lipolytic colonies was increased by this procedure, after twenty-six subcultures the culture appearing to consist entirely of lipolytic mutants. This would appear to have been an example of true selection. Transfer of this strain to fat-free medium, and subsequent subculture, resulted in a completely non-lipolytic variant, as occurred with the originally lipolytic strains. Repeated subculture of the two other originally non-lipolytic strains in fat-containing media, failed to produce lipolytic variants.

The production of lipolytic variants by a process of true selection in media favouring their production has, therefore, been demonstrated inconstantly, and then only with one strain in which they were present

although rarely, in the original culture. The variation observed in other strains would appear to be similar to that described by Dubos (1940) and Topley and Wilson (1946) as selection over a large number of bacterial generations, and is distinct and separate from true bacterial adaptation, which according to Karstrom (1930), Yudkin (1938) and Stephenson (1949) among others, appears without delay, or with only a short lag period (Gale, 1943).

The Effect of the Addition of Fat to Diluted Medium, And of Varying the Constituents of Synthetic Medium, on Growth and Lipase Production.

Fat suspension was added to different types of diluted medium, to determine whether it would have any enhancing effect on the growth of lipolytic Staphylococci, as measured by opacity estimations and a viable counting technique (Part II, 7). No enhancing effects were demonstrated by either of the methods used.

Constituents of the synthetic medium were varied, and duplicate sets put up with and without fat, to determine whether any single constituent was essential for lipase production by Staphylococci, and whether fat would augment their growth when deprived of other nutrients. The only single constituent of the medium found to be essential for lipase production by the strains studied, was cystine (Part II, 8). This observation corresponded with that of Surgella (1937), who had found that cystine was one of the amino-acids essential for the production of enterotoxin by Staphylococci. There was again no

evidence that fat augmented the growth of the *Staphylococci*.

A Study of the Optimal Growth Temperatures of Various Parasitic and Saprophytic Species, In Relation to Lipolytic Activity.

All tests for lipase production had been carried out by growing the organisms at their optimal growth temperature of 37°C. The following experiments were suggested by the frequent references found in published work to the formation of lipases by bacteria living as saprophytes, or at the low temperature range of cold-storage. From the observations of earlier workers it seemed possible that organisms which did not produce diffusible lipase at their optimal growth temperature of 37°C. might do so if they were cultured at lower temperatures.

Strains of *Bacterium* and *Pseudomonas* already studied were, therefore, retested simultaneously with a number of similar species and of species from other genera, isolated from samples of soil, water and milk. Cultures were put up in quadruplicate and incubated at four temperature levels, namely 37°, 30°, 22° and 8°C. (Part III).

Two hundred and eighty-four strains were tested, the majority of which grew at all temperatures of incubation. Only twenty-five of these strains (7.7%), showed lipolytic activity at one or more temperature of incubation. All of these were isolated from

saprophytic sources. In cases where lipase was produced at one temperature level only, this was nearly always found to be the optimal for growth. In those where it was produced at several levels, the degree of lipolysis was nearly always approximately the same. There was, therefore, no evidence to show that bacteria which do not produce diffusible lipase at their optimal growth temperature, will do so when cultured at any other temperature. The restriction of lipolytic activity to saprophytic species or strains belonging to these genera, was confirmed.

The Demonstration of Lipase in Culture Filtrates of Strains of Staphylococci, and a Study of its Immunological Activity.

The wide zone of diffusion of lipase produced on nutrient fat agar by food-poisoning strains of Staphylococci suggested that it might be possible to obtain lipase in a filterable form. These strains were, therefore, cultured under varying conditions, and filtrates tested for lipolytic activity. These conditions were the same as those necessary for the production of staphylococcal toxin (Part IV, 1).

Lipolytic activity in culture filtrates was most satisfactorily assessed using the drop method of assay involving serial dilution of the filtrate (Part IV, 1, iii). The plate and cylinder method used in earlier experiments gave only roughly quantitative results, differences in potency of less than one thousand-fold not being consistently demonstrated by measurement of the diameter

of the zones produced. The areas of activity produced on nutrient fat N.B.S. agar by the drops of filtrate showed differentiation into concentric rings, similar to those produced in the plate and cylinder technique. An arbitrary degree of lipolysis was taken as the titre.

Staphylococcal lipase was found to be relatively stable to heat, boiling for forty to fifty minutes being required to destroy the activity of the filtrate completely. It is difficult to compare these figures with those given by MacFarlane and Knight (1941) for lecithinase, as experimental conditions were different. These workers used dilutions of 1 in 80 in water and borate buffer, and found that boiling for thirty minutes was necessary for complete destruction of lecithinase activity, and that after boiling for ten minutes, 8.0 per cent of the activity persisted when the diluent was water, and 45.0 per cent when it was borate buffer at pH 7.6.

The majority of lipolytic culture filtrates of Staphylococci were found to be stable after storage at 4°C. for nine months. Of those which were not, some lost all activity during the first month of storage, while those of one strain lost all activity after four months of storage.

Preliminary experiments were made to separate the lipase from crude culture filtrates by precipitation with ammonium sulphate and by paper electrophoresis.

These were only moderately successful, and were not continued further (Part IV, 4, iii). The protein material precipitated from the culture filtrates appeared to have almost the same degree of lipolytic activity as the whole filtrate. This, however, could only be assessed approximately by the roughly quantitative plate and cylinder method of assay in use at the time, for estimating the activity of eluates from paper strips, after electrophoresis. Staining of the paper strips proved technically very difficult. By using the method of Nachlas and Seligman (1948) a stained area 8.5 cm. in length could be demonstrated on the paper strip, but no appreciable variation in the depth of staining was apparent.

The Production of Anti-lipolytic Antibodies in Rabbits.

Anti-sera were produced in rabbits by means of injections with living cultures of Staph. albus and with culture filtrates of Staph. albus and aureus, (Part V). These were found to neutralise the lipolytic activity of the homologous filtrate and of a wide range of heterologous filtrates. The maximal neutralisation was given by the undiluted anti-serum, against a range of dilutions of the filtrate, unit volumes of the most potent anti-sera neutralising two hundred and fifty times the amount of lipase contained in that dilution of homologous filtrate taken as the titre. Dilutions of these anti-sera of 1 in 5 and 1 in 10, neutralised sixty and sixteen times

the amount of lipase contained in the titre, respectively.

Staphylococcal lipase differed from some of the other bacterial enzymes which have been used as antigens, notably catalase and urease (Sumner and Myrback, 1950), in that neutralisation by heterologous anti-serum was almost, if not entirely, as marked as with homologous anti-serum. Cross neutralisation has been observed, for example, between the anti-serum to ox liver catalase and ox and horse liver catalase, in which a much greater volume of anti-serum was required to neutralise the heterologous than the homologous catalase (Campbell and Fourt, 1939). The cross-neutralisation tests carried out with staphylococcal culture filtrates and antisera prepared from a relatively small number of strains, gave consistent results, and the evidence obtained suggested that the lipases of Staphylococci are anti-genically homogeneous.

The reaction between lipase and anti-lipase also differed from some other anti-enzyme reactions, in the fact that no pro-zone of inhibition of lipolytic activity with low dilutions of the anti-serum, was observed, such as was encountered by Isaacs and Bozzo (1951), using normal ferret sera and filtrates of Vibrio cholerae.

Normal rabbit serum was used as a control for the immune serum, and no evidence was found of any naturally occurring anti-lipolytic antibody.

SUMMARY AND CONCLUSIONS

The results of the experimental work can be very briefly summarised as follows:

- 1) A solid medium is the more sensitive for demonstrating diffusible lipase produced by growing bacteria.
- 2) Among parasitic bacteria the only genus in which the power to produce diffusible lipase is widespread is the Staphylococci.
- 3) The production and demonstration of diffusible lipase was not found to be influenced by:
 - a) the anaerobic cultivation of aerobes;
 - b) alteration of the oxidation-reduction potential of the medium by the addition of sodium thioglycollate;
 - c) the addition to the medium of sodium taurocholate or horse serum.
- 4) Diffusible lipase is produced only when the bacterium is grown under optimal conditions.
- 5) The amino-acid cystine is an essential metabolite for lipase production.
- 6) The production of diffusible lipase by Staphylococci does not appear to be specifically related to any other characteristic.
- 7) The formation of lipolytic mutants was observed in two non-lipolytic strains of Staphylococci. These were increased in number by subculture in fat-containing medium.
- 8) All enterotoxigenic strains of Staphylococci tested showed marked lipolysis.
- 9) Most strains of Staphylococci which produced diffusible lipase on solid medium showed lipolytic activity in their culture filtrates, provided that the cultures were grown in conditions favourable to the production

of staphylococcal toxins.

- 10) No purification of lipase was obtained by precipitation of protein from the filtrate with ammonium sulphate followed by paper electrophoresis.
- 11) Staphylococcal lipase in culture filtrates is fairly stable to heat and to storage....
- 12) Immune sera produced in rabbits neutralised the lipolytic activity of homologous and heterologous filtrates to a comparable degree. No evidence of naturally occurring antilipolytic antibody was found in normal rabbit serum.

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