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THE BIOSYNTHESIS OF METHYL KETONES
WITH SPECIAL REFERENCE TO THEIR
PRESENCE IN CHEDDAR CHEESE

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presented for the Degree
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Massey University of Manawatu

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PREFACE

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Early work on the formation of methyl ketones as artifacts during the steam distillation of Cheddar cheese and milk fat has been published (J. Dairy Research (1963). 30, 161). A note on the origin of methyl ketones using radioactive milk fat was published in co-authorship with Dr. J.C. Hawke (Nature (1963). 197, 1276). A paper will be presented to the N.Z.I.C. Conference in August 1964 on the relationship of milk fat synthesis to the methyl ketones found in steam distillates of milk fat. Early results on the oxidation of fatty acids by Penicillium roqueforti were communicated to the N.Z.I.C. Conference (August, 1963) and more recent work will be presented, in co-authorship with Dr. J.C. Hawke, at the 6th International Biochemical Congress (July, 1964) in New York.

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I N T R O D U C T I O N

Investigations during the past 60 years have led to a fairly detailed knowledge of the chemical substances produced during the ripening of various cheeses but, apart from the established importance for flavour of the methyl ketones in mould ripened cheese (Morgan & Anderson 1956) no major advance in understanding the relationship between composition and flavour has been made. This is particularly true of Cheddar cheese since there exists a range of modifications of the basic Cheddar cheese flavour which appeal to observers in different ways.

The chemical nature of Cheddar cheese flavour has been investigated for many years. Although a large number of compounds have been reported disagreement still prevails concerning the significance of any given compound or class of compounds (carbonyls, fatty acids, hydrogen sulphide etc.) to the flavour. The concept held by the earliest investigators into the flavour of Cheddar cheese e.g., Suzuki, Hastings & Hart (1910), was that a single compound is responsible for the typical flavour of Cheddar cheese. Dacre (1955) also came to this conclusion in his investigations although he was unable to identify the individual flavour principle. Similarly Patton (1961) put forward the view that there is a single compound which, as such, has cheeselike character. Thus according to Patton all cheeses (Cheddar, Blue, etc.), have a basic cheesiness, the distinctive flavour of the various varieties of cheese being due to compounds giving overtones of flavour. Mulder (1952) and Kosikowski & Mocquet (1958) suggested, however, that Cheddar flavour is due to a mixture of compounds derived from degradation of fat, protein and lactose. Individually these components have

flavour but not necessarily cheese flavour. Collectively, when in an appropriate balance, the resulting flavour is that of typical cheese flavour. Support for this "component balance" hypothesis has been given by Harper (1959), Mabbitt (1961) and McGugan (1963) in recent reviews on the chemistry of Cheddar cheese flavour. They conclude that characteristic flavour development in Cheddar cheese is related to simultaneous proteolytic and lipolytic activities which must proceed interdependently at definite rates for optimum flavour formation.

Failure of attempts to attribute the characteristic Cheddar flavour to any of the major constituents of ripe cheese, such as amino acids, and the conclusion of Dacre (1955) that the components of typical flavour are volatile and are present in Cheddar in concentrations of only a few p.p.m., have led investigators recently to examine more closely the minor volatile components of cheese, particularly fatty acids and carbonyl compounds that might originate from the fat phase.

THE IMPORTANCE OF MILK FAT IN CHEDDAR FLAVOUR FORMATION

Mabbitt and Zielinska (1956) observed that no typical flavour develops in "Cheddar" cheese made from skim milk, suggesting that milk fat is the substrate for typical cheese flavour. They found that the typical Cheddar flavour was soluble in fat solvents and was concentrated in the fat phase of cheese. These observations have been confirmed by a number of investigators (Sjoström 1958; McGugan, 1963; Patton 1963).

It has also been found that increasing lipolysis in the cheese by the addition of enzymes or by homogenisation can improve the flavour (Babel & Hammer, 1945). Of especial interest is the improvement obtained in the flavour of pasteurised milk cheese by inoculation of the cheese milk with Geotrichum candidum 18 hours before cheesemaking, the improvement being accompanied by an increase in concentration of C₆ and higher acids (Irvine, Bullock & Sproule, 1954).

It follows that if lipolysis is important in cheese flavour formation, then so probably is the composition of the milk fat. This can be varied by altering the feed, plane of nutrition, environment and the stage of lactation (Jack & Smith, 1956). Thus marked seasonal variations have been found in the quantitatively important volatile fatty acids in N.Z. butterfat. Hansen & Shorland (1952) found that at the beginning of lactation the C₈ - C₁₄ acids comprised 18.6 moles % of the total fatty acids but had increased to 26.9 moles % 6 months later. Similarly Keeney (1956) showed for U.S. butterfat that there were wide fluctuations from 24.24 to 33.55 in the Reichert Meisel value (arbitrary measure of the C₄ and C₆ acids) and that the Polenske values (arbitrary measure of the volatile insoluble fatty acids) varied from 1.12 to 2.95. Such studies may explain why the quality of Cheddar cheese is highest in the Northern Hemisphere when summer milk is used (Kosikowski, 1957; Mabbitt, 1961). Similarly European Gruyère reaches finest quality when made from Autumn milk.

FLAVOURFUL SUBSTANCES IN CHEDDAR CHEESE

Free Fatty Acids

The importance of fatty acids to the flavour of Cheddar cheese is not yet established. Berridge (1953) found acetic acid but neither propionic nor butyric acids, confirming the report of Hiscox (1941) that lipolysis was not marked in Cheddar cheese made from pasteurised milk and that acetic acid constituted the major part of the volatile acids. Windlan (1955) found only acetic acid in American Cheddar in the early stages of ripening. Lawrence (1962) in a detailed investigation of N.Z. Cheddar cheese detected large but variable amounts of acetic acid, smaller amounts of butyric acid but no significant quantities of acids higher than butyric. Other workers however have presented somewhat contrary results. Peterson & Johnson (1949) reported an increase of acetic, C₆, C₈ and C₁₀ acids during Cheddar cheese ripening. Butyric acid, present in high level at the start of the ripening period, did not however further increase. Kristoffersen, Gould & Harper (1959) found that propionic, butyric and higher acids all increased during ripening.

Recently Patton (1963) has produced results that tend to confirm the hypothesis that milk fat is the source of Cheddar aroma formation and that lipolysis of the fat is the mechanism by which it occurs. Patton concludes that the volatile acids (acetic, butyric, hexanoic and octanoic) constitute the foundation or "sine qua non" of Cheddar aroma, confirming the findings and opinions of some earlier workers e.g. (Suzuki, Hastings &

Hart, 1909; Peterson & Johnson, 1949; Kristofferson & Gould, 1960). Patton found acetic acid to be the dominant volatile acid and presumably of substantial importance to the unique aroma of the cheese, since in its absence the mixture of higher fatty acids ($C_4 - C_8$) simply had a rancid aroma. The origin of this acetic acid has yet to be established.

Hydrogen sulphide

Kristoffersen & Gould (1960) found a significant statistical correlation between hydrogen sulphide concentration and Cheddar flavour, in particular between the characteristic flavour and the relative concentrations of free fatty acids and hydrogen sulphide. Subsequently however, Kristoffersen (1961) reported that the flavour sensation provided by some Cheddar cheeses was not always related to the level of hydrogen sulphide, particularly in the early stages of curing. Lawrence (1963) could find no simple relationship between hydrogen sulphide concentration and the characteristic Cheddar flavour of N.Z. Cheese. He considered that it was possible, however, that hydrogen sulphide combines with some other product of ripening, perhaps with carbonyl, carboxyl or ester groupings, with which it is known to form addition compounds (Connor, 1943), and that it is this combination which is responsible for the typical Cheddar flavour.

Methional

One hypothesis of Cheddar cheese flavour formation put forward by Keeney and Day (1957) suggests that a slow

chemical interaction, over the long period of ripening, between amino acids and dicarboxyl compounds (Strecker degradation) could lead to the production of flavourful aldehydes containing one carbon atom less than the original amino acid. Thus methionine would give rise to methional (3-methylthiopropional) which was claimed to have a cheese-like odour. Other investigations (Oro, Guidry & Zlatkis, 1959; Jackson, 1958; Keeney, 1961) have stressed the importance of methional in Cheddar cheese flavour, but this has been disputed in turn by Witting & Batzer (1957), Walker & Harvey (1959) and Kosikowski (1961). The subject of methional was discussed at length at a symposium on flavour chemistry sponsored by the Campbell Soup Company (1961) but no agreement could be reached on its importance in Cheddar flavour.

Methyl Ketones

The significance of methyl ketones in relation to the flavour or aroma of cheeses has been investigated by a number of workers. It appears that these substances are important constituents of the flavour of mould-ripened cheeses but their contribution to the flavour of other types of cheese is not so clearly established. Harvey and Walker (1960) isolated a range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{11} by steam distillation of Cheddar cheese at atmospheric pressure and considered that their time of first appearance and increase in concentration were correlated with the increase in intensity of the Cheddar flavour as the cheeses matured. Similarly Jackson (1958), using steam distillation at atmospheric pressure to concentrate the

Cheddar flavour, found 2-heptanone, 2-nonanone, butyradlehyde and diacetyl by subsequent gas chromatographic examination. The peaks corresponding to these carbonyls seemed, however, to have no relation to cheese aroma. Other workers (Day, Bassette & Keeney, 1960; Patton, Wong & Forss, 1958; Day & Keeney, 1958) isolated a range of odd-numbered methyl ketones, as well as other carbonyls, from Cheddar cheese by distillation under reduced pressure at a temperature between 40° and 50°.

In contrast, however, to the above findings it is significant that when the extraction of carbonyls was carried out at room temperature (Bassett & Harper, 1958; Kristoffersen & Gould, 1959) no trace of methyl ketones other than acetone was obtained. Bassett & Harper (1958) further observed that the yield of 2-nonanone from Blue cheese was increased by heating the cheese slurry for 10 min. at 100°. This suggests that the methyl ketones isolated by other workers might have been artifacts produced by the elevated extraction temperatures. This is a criticism which could apply to all extraction methods involving heating of the cheese slurry since if methyl ketones were being formed as artifacts at 100°, it was also probable that a similar breakdown occurred to some extent at the lower temperatures (40-50°) employed by some workers.

Carbinols

In a gas chromatographic study McGugan & Howsham (1962) found that the major components of the neutral volatiles from Cheddar cheese were 50% 2-butanol, 9% isopropyl alcohol, 5% pentanone, 4% acetone with smaller amounts of some 40 to 50 volatile components. One

cheese with a slightly fruity flavour showed over 80 components, Scarpellino (1961) also reported the presence in Cheddar cheese of 2-butanone, 2-butanol and ethyl alcohol in relatively large amounts confirming that carbinols are major metabolic products of cheese ripening.

FLAVOURFUL SUBSTANCES IN BLUE CHEESE

Although Currie (1914) considered that the C_6 , C_8 and C_{10} acids gave Blue cheese its flavour, Starkle (1924) concluded that the characteristic aroma in the ripening of cheese by fungi was due to methyl ketones. This was also the opinion of Hammer & Bryant (1937); Berridge, Hiscox & Zielinska (1953); Nesbitt (1953) and Proks, Dolezalek & Pech (1958).

Patton (1950) and Morgan & Anderson (1956) identified methyl ketones with an odd number of carbon atoms from C_3 to C_{11} in steam distillates obtained by distillation of Blue cheese at atmospheric pressure. The possibility that part or all of these methyl ketones were artifacts of the steam distillation was examined by Schwartz & Parks (1963). However using an extraction procedure not involving heat they showed that the odd numbered methyl ketones from C_3 to C_{15} were present in mature Blue cheese. The major contribution of methyl ketones to the flavour of Blue cheese is almost proven although Graham (1958) obtained only a fair correlation between flavour scores of Blue cheese and volatile acidity and neutral carbonyl compounds.

Methyl carbinols corresponding to the C_5 , C_7 and C_9

methyl ketones have been identified in Blue cheese (Jackson & Hussong, 1958; Coffman, Smith & Andrews, 1960) as has also ethyl alcohol (Bavisotto, Rock & Lesniewski, 1960). Since the addition of ethyl alcohol to homogenised milk impregnated with P. roqueforti caused an intensification of Roquefort aroma (Sjöström & Malm, 1952) it is possible that these alcohols combine with the free acids found in cheese to form esters, which may contribute to the flavour.

It does not necessarily follow that because both Cheddar and Blue cheese have been found to contain methyl ketones and some of the corresponding carbinols that these compounds are the cause of their basic cheesiness. However it would appear that certain biochemical mechanisms in the ripening of both Cheddar and Blue cheese are similar.

The flavour of Blue has been no easier to reproduce than the more delicate flavour of Cheddar cheese and the fact that no satisfactory cheese flavour concentrate (natural or synthetic) has yet been reported shows the need for continued investigation. A full understanding of any one cheese flavour might be of great help in elucidating the flavour of all varieties.

SIMULATION OF CHEDDAR FLAVOUR

Generally it is considered positive proof that a compound is a part of cheese flavour if its addition to cheese or to a synthetic bland base will reproduce or

enhance the flavour. Silverman & Kosikowski (1953) found that the addition to a bland cheese of a balanced quantity of pure fatty acids normally found in cheese resulted in a distinct butyric-rancid sensation. When however amines and amino acids were added as well as fatty acids, a Cheddar-like flavour resulted although it was not a complete Cheddar flavour. Day, Bassette & Keeney (1960) added various mixtures of ketones and fatty acids to bland cheese curd but the flavours produced were sharp and musty. Walker (1961) confirmed this finding but found that addition of thioacetamide, a source of hydrogen sulphide, much improved the flavour. This observation has not yet however been confirmed in other laboratories. The view of Mabbitt (1961) that the metabolism of sulphur compounds in Cheese may be significant in flavour formation has been strengthened by the recent observation of Scarpellino (1961) that an aqueous solution of 2-butanone, butyric acid, acetic acid and methionine gave an aroma akin to that of Cheddar cheese.

MECHANISMS OF METHYL KETONE FORMATION

An appraisal of previous investigations into the chemical compounds contributing to cheese flavour led to the conclusion that a study of methyl ketones from lipids, and in particular their mode of formation, was the most promising avenue of approach.

The first hypotheses to be advanced to explain the detection of methyl ketones in steam distillates of dairy

products containing milk fat were those of Wong, Patton & Forss (1958). They considered that 2-pentanone and 2-heptanone found in steam distillates from evaporated milk could possibly result from the decarboxylation of the corresponding β -keto acids. These β -keto acids might occur in milk as:

- (1) Intermediates in the synthesis of fatty acids from acetate by the mammary gland.
- (2) Degradation products of fatty acids by β -oxidation.
- (3) Deterioration of the milk lipids through attack by atmospheric oxygen.

They considered the last possibility to be remote in evaporated milk or heated cream which contain a wealth of reducing substances.

Although a considerable amount of research has been carried out on the oxidation of milk fat (e.g. Forss, Dunstone & Stark, 1960; Day & Lillard, 1960), a clear distinction has not always been made between the effect of heating and the effect of oxidation. The first study on thermal degradation of milk fat was that of Patton & Tharp (1959), in which they stripped the fat with steam for a period of 3 hr, holding it at a temperature of 200°C and a pressure of 0.1 - 0.25 mm Hg. They identified an homologous series of methyl ketones containing the n-alkyl members with odd numbers of carbons from C₃ to C₁₅, which suggested that methyl ketones found in steam distillates of dairy products containing milk fat were artifacts. Nawar, Cancel & Fagerson (1962)

thought that some form of oxidation might be involved since bleaching of color was observed in milk fat upon heating. However, the loss of colour by heat appeared to proceed via a different mechanism than the bleaching normally produced by intensive oxidation of milk fat. No oxidized flavour was detected when the fat was heated to the point of discolouration in the absence of oxygen and moisture. Gas chromatographic patterns of the heated fat were markedly different from those of milk fat oxidized at low temperatures which indicated that aldehydes predominated as products of oxidative mechanisms, whereas ketones were the major components formed by heat.

The origin of methyl ketones in cheese

The weight of opinion up to 1962, despite the work of Patton and his coworkers, was that the methyl ketones detected in steam distillates from Cheddar cheese were derived by β -oxidation of the C_4 to C_{14} fatty acids of milk fat (Sjöström, 1959; Harvey & Walker, 1960; Mabbitt, 1961). For example the possibility of artifact formation was considered by Harvey & Walker (1960) but they concluded that the methyl ketones obtained in their work were not artifacts formed during the distillation but were present as such in Cheddar cheese.

THE BIOSYNTHESIS OF THE FATTY ACIDS OF MILK LIPIDS

The possibility however that precursors of methyl ketones might arise as intermediates in milk fat synthesis led to a study of the factors involved in the biosynthesis

of fatty acids in the mammary gland. The two main problems of milk fat secretion are its origin and its difference in composition from body fats. Whereas ox depot fatty acids consist chiefly of stearic, palmitic and oleic acids, cow's milk fat contains about 10% of the C_4 to C_{12} acids (see Hilditch, 1956).

For some years it has been appreciated that the fatty acids of milk triglycerides arise from two distinct sources - from blood plasma lipids and by direct synthesis in the mammary gland. Since cow's blood contains about 0.3% total lipids the earliest work consisted of attempts to find what fraction of the blood lipids gives rise to milk fat. Graham, Jones & Kay (1936) found that there was sufficient arterio-venous difference in total blood fatty acids (i.e. free and combined) to account for all the fat in milk and this conclusion was confirmed by other workers, e.g. Shaw, Powell & Knodt (1942). Riis, Luick & Kleiber (1960), considered that some 50% of the fatty acids of the milk came from colloidal fatty material in the plasma which was supported by the finding of Hardwick, Linzell & Mephram (1963) that about 30% only of the non-volatile fatty acids could be synthesised from acetate by isolated perfused goat udder. Barry, Bartley, Linzell & Robinson (1963) concluded that the fatty acids taken up by the goat mammary gland from the blood were principally palmitic and stearic acid.

If the blood is accepted as the source of milk fat there remains its peculiar fatty acid composition to be accounted for. Achaya & Hilditch (1950) suggested that the short chain acids are formed in the udder by degradation, from the omega end of the molecule, of oleic or other unsaturated acids combined in triglycerides. However

the function of acetate in the formation of milk fat was directly demonstrated by the finding of Popják & Beeckmans (1950) that acetate carbon was incorporated into the mammary fat of pregnant rabbits. This led to an experiment (Popják, French & Folley 1951) on the metabolism of 1-¹⁴C acetate by the lactating goat when about 10% of the dose was secreted into the milk in the form of fatty acids, the short-chain acids having the highest specific activity. Cowie et al (1951) considered that the decrease of specific activity of the milk and udder fatty acids with increasing chain length indicated that the short chain acids could not have arisen to any considerable extent by the direct degradation of long chain acids as required by the theory of Hilditch. However, as Glascock (1958) pointed out, although the weight of evidence is now against this theory it was not disproved. Many substances have more than one precursor and the specific activity data of Popják et al (1951) does not appear to exclude their being formed in part from long chain acids by some mechanism other than that postulated by Hilditch.

The conclusion of Popják et al (1951) that milk-fatty acids arise both from acetate and from a circulating C₄-compound agrees with the finding of Shaw & Knodt (1941) that β-hydroxybutyrate is taken up by the lactating udder. Popják et al considered that fatty acids are formed in part by a conversion of β-hydroxybutyrate to butyrate followed by condensation with acetate. Support for this hypothesis was given by Kumar, Lakshmanan & Shaw (1959) who found that in the milk secreted by an isolated udder perfused with 1-¹⁴C-β-hydroxybutyrate all the acids up to capric (C₁₀) were labelled. The specific activity

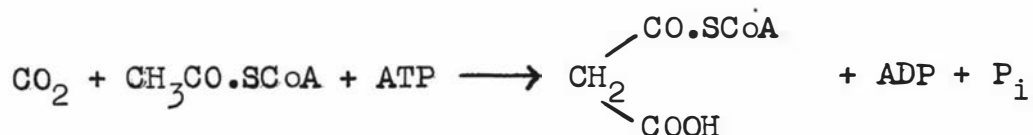
of these acids increased with increasing chain length up to C₈ acid, whereas lauric and longer chain acids had negligible specific activity. In the presence of an adequate supply of acetate, butyrate should therefore be as good a precursor of milk fatty acids as either β -hydroxybutyrate or acetate itself. Black, Kleiber & Brown (1961) found however that much less labelled butyrate than acetate was incorporated into milk fatty acids by the living cow but that there was substantial incorporation of butyrate into lactose. Thus while Kumar, Lakshmanan & Shaw (1959) have demonstrated that β -hydroxybutyrate can undoubtedly be metabolised to yield fatty acids of chain length up to 10 carbon atoms, the intermediary pathway is not clear and, as Glascock (1958) has pointed out, is unlikely to involve butyrate. Indeed recent work by Annison, Leng, Lindsay & White (1963) has shown a high incorporation of butyrate carbon, after its infusion into the portal veins of sheep, into β -hydroxybutyrate of the blood.

Propionate is not apparently incorporated to any great extent into milk fatty acids, though propionate can be the precursor of the odd-numbered fatty acids in milk fat and, following its degradation, to even-numbered fatty acids. (James, Peeters & Lauryssens, 1956).

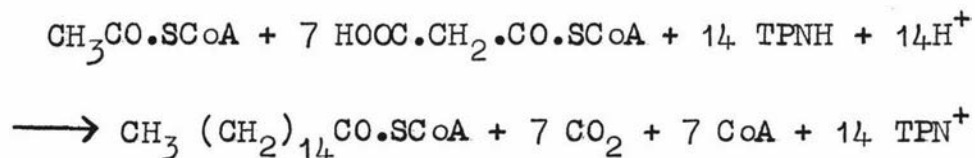
Hilditch (1956) in view of the work of Popják et al (1951) put forward an alternate pathway, without experimental evidence, that envisioned milk fat synthesis as a process of fatty acid transacylation onto preformed triglyceride molecules which were previously absorbed from the blood. More recently McCarthy et al (1960) have proposed that two mechanisms might be involved in milk fat synthesis : alteration of pre-existing triglyceride molecules and

supplemental synthesis from non-lipid precursors. Luick (1961) put forward the hypothesis that milk fat is synthesised "de novo" within the mammary gland from pools of free fatty acid and glycerol but emphasised that this did not exclude the simultaneous formation of milk fat from plasma glyceride via mechanisms involving fatty acid exchange, substitution or rearrangement within the mammary gland. The conclusions of Luick & Lucas (1962) and of Patton et al (1962) that plasma lipids are hydrolysed in the udder to provide such a pool of free fatty acids provides additional support for this hypothesis.

Following the early work of Popják, Folley and their collaborators from 1950 onwards, it was generally considered that the synthesis of saturated fatty acids from acetate was probably a reversal of β -oxidation. The studies of Wakil, Brady and their coworkers since 1958 however showed that a source of CO_2 was of fundamental importance in fatty acid synthesis by particulate-free preparation of pigeon liver and chicken liver. This work and other related studies (reviewed by Wakil, 1961) showed that this biosynthetic pathway was different from a reversal of β -oxidation. Fatty acid chains of up to 16 carbon atoms are now known to be elaborated from acetyl CoA which condenses with HCO_3^- ion to yield malonyl CoA in the presence of ATP, Mn^{++} ions and acetyl CoA carboxylase (a biotin-containing enzyme).

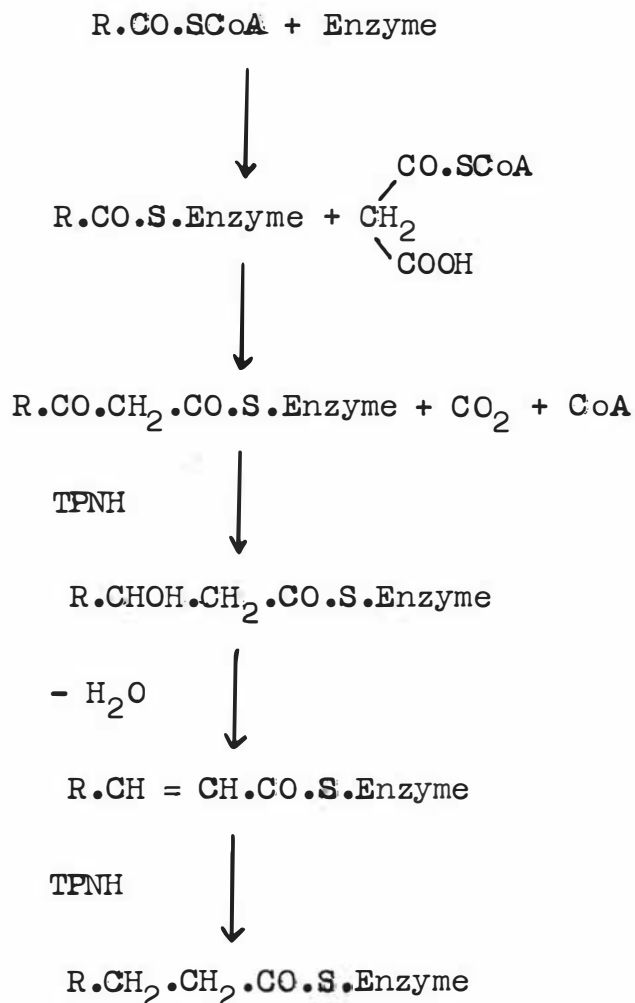


Malonyl CoA condenses in turn with acetyl CoA to yield by reaction with reduced triphosphopyridine nucleotide (TPNH), CO₂, CoA and a saturated fatty acid. Thus successive condensations yield palmitic acid, the stoichiometry being expressed as follows:



The malonyl CoA system has now been characterised in a variety of animal tissues and microorganisms. Usually long chain fatty acids are formed in these systems, a notable exception being preparations from mammary tissue. Ganguly (1960) found that such extracts synthesised both the long and short chain acids, the relative proportions of C₄, C₆ and (collectively) the C₈ - C₁₆ acids synthesised by the mammary gland system being similar to those present in cows' milk fat.

Although none of the intermediates in the biosynthetic sequence has been definitely identified, the interaction between acyl CoA and malonyl CoA as a concerted condensation-decarboxylation has been proposed by Vagelos and Alberts, (1960) since decarboxylation at this point would pull the reaction in favour of condensation. This hypothesis was initially based on the observation that CO₂ can exchange with a carboxyl group of malonyl CoA in the presence of saturated acyl CoA. The acyl intermediates are considered to be enzyme bound (Lynen, 1961).



Dils & Popják (1962) showed the malonyl CoA route to be of major importance in fatty acid synthesis by soluble preparations of lactating rat mammary gland. Several intermediates of the citric acid cycle, formerly thought necessary to stimulate fatty acid synthesis, were no longer required. The principal products of synthesis were the saturated n-fatty acids C₈ - C₁₈, together with a small proportion of oleic acid, although, compared with milk fatty acids, a somewhat

greater proportion of lauric and myristic acids was produced. Abraham, Matthes & Chaikoff (1961) carried out similar work with lactating rat mammary gland but found an absolute requirement for citrate, the stimulating effect of free malonate being observed only in the presence of citrate. The fatty acids synthesised were qualitatively similar to those obtained by Dils & Popjak although the proportion of C₈ - C₁₂ acids produced was much greater.

MICRO-ORGANISMS IN CHEDDAR CHEESE

The alternative hypothesis put forward by Wong et al (1958) to explain the presence of β -keto acids in dairy products containing milk fat was a degradation of fatty acids by β -oxidation. If methyl ketones are formed by micro-organisms in cheese and are contributing to the flavour it seemed logical to examine any relationships reported between flavour and these micro-organisms.

Numerous attempts by earlier workers to find a correlation between the composition of the microflora of cheese and the time of appearance of flavour have not been entirely successful (e.g. Sherwood, 1939). Robertson (1961) in an investigation of Cheddar cheese from 4 different countries has shown that apart from quite large differences between individual cheese, the flora of the cheese of one country tends to be quite different from that of another. Certain features such as the proportion of L.casei in the flora are uniform

from one country to another but even with this species the distribution of the strains which are unable to ferment melezitose show a country to country variation.

Possible reasons for the differences in flora between the cheese of different countries are the composition of the milk, its flora and the type of bacteria gaining admission during cheesemaking. These variables in turn depend on such complex factors as the type of pasture, climate, standards of hygiene and methods of manufacture. A more tangible influence is the presence or absence and degree of heat treatment of the milk used for cheesemaking. Many investigators have found that the rate of flavour development in pasteurized milk Cheddar cheese is slower and the final flavour is milder than in raw milk cheese, e.g., Kristoffersen & Cole, 1960. This effect has usually been attributed to the decrease in numbers of micro-organisms and inactivation of milk enzymes as a result of the heat treatment.

Notwithstanding marked variations in the bacterial flora found in the cheese examined from 4 different countries. Robertson (1961) concluded that all the cheese examined had what could be described as a basic Cheddar flavour. It would seem therefore that the enzymes responsible for this basic flavour must be common to all cheese. They could originate from the rennet, milk or micro-organisms.

The inoculation of cheese milk with certain cultures, particularly lipolytic organisms such as certain strains of lactobacilli (Sherwood, 1939), pseudomonads, achromobacteria and flavobacteria (Stadhouders & Mulder, 1957) and micrococci (Robertson & Perry, 1962) have been

found to enhance flavour production in Cheddar cheese.

It has recently been demonstrated that bacteria which are present in relatively small numbers may nevertheless be of importance in cheese ripening. Stadhouders & Mulder (1957) have shown that bacteria, which may multiply in the milk during storage but which do not grow in the cheese, can produce lipolysis of the cheese fat. Furthermore this lipolysis may still occur, though on a reduced scale, even if the cheese milk is pasteurised, since the lipolytic enzymes are sufficiently heat stable to retain some activity (Stadhouders, de Vries & Mulder, 1959).

In view of the above findings of Robertson (1961) the bacterial flora actually present in the cheeses appear not to make the major contribution to flavour production. However, organisms that are present in the original milk flora or fall into the vat during cheesemaking could after death leave active enzyme systems that contribute to flavour production. This is substantiated by the work of Perry & McGillivray (1964). They successfully designed an apparatus to exclude bacteria from the vat during cheesemaking and by using initially bacteria free milk were able to assess the overall importance of streptococci and strains of lactobacillus and micrococcus in cheese ripening. It was significant that when "aseptic" cheeses were manufactured under controlled conditions, no flavour developed in the cheeses except in the control cheeses when the lid of the "aseptic" vat was opened and adventitious bacteria from the atmosphere allowed to fall in.

Presumably the organisms producing Cheddar cheese flavour are natural components of the microbiological flora in the factory. Olson & Hammer (1934) found that up to 50% of the microbial flora of a cheese factory examined were fungi and yeasts. These have also been isolated from Cheddar cheese in a viable state (Robertson, 1964). They would be present in milk churns and pipe lines despite average cleanliness and would be able to grow in milk during storage. Pasteurization might destroy the growing organisms and spores but lipolytic enzymes have been shown to be very resistant to heat (Stadhouders, de Vries & Mulder, 1959). The fungi and yeasts which also fall into the vat during cheesemaking may be important, particularly in view of the work of Stadhouders & Mulder (1957) reported above.

The high proteolytic activity of fungi and yeasts (Proks, Dolezalek & Pech, 1959) is possibly also of importance since their ability to degrade the protein-phospholipid complex in the so-called membrane of milk fat globules might allow the globules themselves to be attacked more readily.

Although the microbiological flora of Cheddar cheese is predominately bacterial a study of the β -oxidation of fatty acids by any of the numerically important bacterial species isolated from Cheddar cheese did not seem logical. The microbiological species responsible for Cheddar flavour are not known, although the indications are that they are lipolytic. However no lipolytic bacterium has been reported capable of oxidising fatty acids to methyl ketones. Since on the other hand fungi are in general more lipolytic than bacteria and are known to form methyl ketones from lipids, a detailed investigation

of the oxidation of lipids by Penicillium roqueforti (a fungus known to be responsible in part for the flavour of Blue cheese) was undertaken. While it is unlikely that fungi would grow in the anaerobic environment and high local concentration of carbon dioxide (Robertson, 1957) to be found in the interior of ripening Cheddar cheese it was thought possible that viable spores might secrete extracellular enzymes capable of oxidising fatty acids derived from milk fat triglycerides to β -keto acids and by decarboxylation give the corresponding methyl ketone.

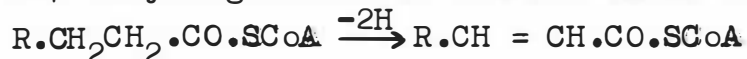
FATTY ACID OXIDATION

Several workers (e.g. Green, 1954) have demonstrated that fatty acids are oxidized via a pathway very similar to the β -oxidation scheme first postulated by Knoop in 1904. There are five basic steps in this cycle:

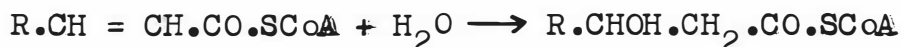
1. Esterification of the fatty acid to a thiol ester of Coenzyme A:



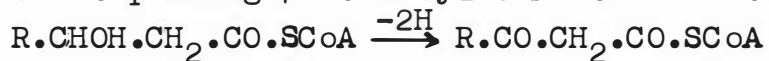
2. α - β Dehydrogenation of the CoA ester of the fatty acid:



3. Addition of water across the double bond of the unsaturated acyl CoA ester:



4. Oxidation of the β -hydroxyacyl CoA ester to the corresponding β -ketoacyl CoA derivative:



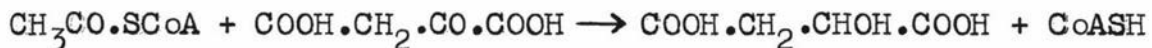
5. Thiolysis by CoASH of the β -ketoacyl CoA ester to acetyl CoA and a new fatty acyl CoA ester:



The net result of these five steps is the splitting out of acetic acid in the form of its Coenzyme A ester and the formation of a new fatty acyl CoA ester with two carbon atoms fewer than the parent ester. The cycle then repeats itself until the fatty acid chain is entirely degraded to units of acetic acid.

The β -oxidation cycle refers to this sequence of five steps which is repeated as many times as the number of paired carbon atoms in the chain. Since in general fatty acids of biological origin contain an even number of carbon atoms and have a straight chain, β -oxidation leads to the scission of the fatty acid to the appropriate number of acetic acid residues.

The condensation of acetyl Coenzyme A, the end product of the β -oxidation sequence, with oxalacetate to form citrate (Stern & Ochoa, 1951) is of considerable significance.



First, it leads to the release of Coenzyme A which is then made available for a repeat of the β -oxidation cycle. The concentration of coenzyme in vivo is limited and unless the coenzyme is being turned over the cycle would

stop. Second, the citric acid formed can be oxidized to CO_2 and H_2O through the citric acid cycle. By means of this cyclical oxidation sequence the fatty acid oxidation cycle is channelled into the main mitochondrial stream.

Oxidation of Fatty Acids with an Odd Number of Carbon Atoms

The mitochondrial system oxidizes fatty acids with odd or even numbered carbon atoms at a similar initial rate (Grafflin & Green, 1948), and this has been confirmed by studies with the isolated enzymes of the fatty acid oxidation cycle (Mii & Green, 1954). The end products of the oxidation of odd numbered fatty acids are acetyl CoA and propionyl CoA. If the mitochondria like those of the liver are capable of oxidizing propionate to CO_2 and H_2O , the odd numbered fatty acids pose no problem. However, where this is not the case as for heart mitochondria, the oxidation of the odd numbered acids proceeds with great difficulty (Grafflin & Green, 1948). This could be largely the consequence of the immobilization of CoA in the form of propionyl CoA which is metabolically inert.

OXIDATION OF FATTY ACIDS BY FUNGI

The enzymes responsible for the oxidation of fatty acids in mamalian and plant tissues have been thoroughly investigated (Green, 1954; Lynen, 1955; Stumpf & Broadbear, 1959). In the case of fungi, in which a

variety of effects of fatty acids on growth and metabolism have been observed, the knowledge of enzymatic processes is still comparatively undeveloped. Although it has generally been assumed that the same enzyme systems occur in fungi as in animal and plants tissues, there is little experimental evidence to support this assumption. This paucity of information may be due in part to certain technical difficulties that might be encountered in work with fatty acids.

(a) In low concentrations (i.e. up to 10 μ moles per ml) it is difficult to analyse for suspected intermediates that might accumulate in small amounts.

(b) Toxicity and low solubility, on the other hand, put an upper limit on the concentrations that may be used.

In animal and plant tissues, oxidation of fatty acids to CO_2 and H_2O (i.e. complete oxidation) is a function reserved exclusively for mitochondria. Since the enzymatic principles which underlie the basic mitochondrial functions have generally applicability regardless of the source of the mitochondria, it would be anticipated that the pattern of fatty acid oxidation to CO_2 and H_2O would be much the same in all animal and plant tissues. Although there is insufficient evidence of the invariant link of fatty acid oxidation to organized particles in microorganisms, wherever the process has been studied it has been found to correspond closely with the equivalent process in animal and plant tissues.

Some steps of β -oxidation were confirmed substantially for *P.glaucum* by Thaler and co-workers who obtained methyl ketones from α - β unsaturated acids (Thaler & Eisenlohr, 1941), from β -hydroxy acids (Thaler & Geist, 1939), and from β -keto acids (Thaler & Stahlin, 1945).

Mukherjee (1951) found that fatty acid dehydrogenase activity of *Penicillia* and *Aspergilli* increased from C_4 to C_{10} with C_{16} and C_{18} acids showing no dehydrogenase activity. Contradictory results were obtained however by Franke & Heinen (1958) who isolated a constitutive dehydrogenase enzyme with maximum activity for C_{16} and C_{18} fatty acids and very low activity for C_6 and C_8 acids.

The ability of *Penicillia* to oxidise C_8 and C_{10} acids to methyl ketones was first noted by Starkle (1924). In 1951 Mukherjee claimed that only the genera *Penicillia* and *Aspergilli* were able to form methyl ketones from fatty acids but Franke & Heinen (1958) later showed that the methyl ketone forming ability of fungi was general if not ubiquitous. Of 39 fungi tested, 2 *Phycomycetes*, 1 *Ascomycetes* and 6 *Fungi Imperfecti* did not give methyl ketones. Not all species of the same fungus however formed methyl ketones.

Saturated fatty acids are utilised by some fungi without any trace of ketone formation, and furthermore when ketone is formed the yields are not quantitative. The immediate precursors of the ketones are believed to be the corresponding β -keto acids. Two mechanisms thus compete for the β -keto acid in fatty acid oxidation (a) oxidation to CO_2 and H_2O (b) decarboxylation to methyl ketone.

In methyl ketone formation it is considered that the thiolase reaction is inhibited, the β -ketoacyl-Coenzyme A ester being deacylated to the free β -keto acid which is subsequently decarboxylated.

conclusion of Thaler & Geist that the yield of methyl ketone reached a maximum just before visible mycelium appeared. Apart from the complications due to culture growth the above investigations were also not carried out in shaken cultures. Under such conditions abnormal respiratory conditions are certain to exist (Foster, 1949).

Gehrig & Knight (1963), using a medium not containing a nitrogen source and with 3-C¹⁴ octanoic acid as sole carbon substrate, found that spores oxidised low concentrations (1 μ mole) without formation of methyl ketone although small amounts of radioactive 2-heptanone were detected when large amounts of substrate (20 μ moles) were used. No quantitative details of the oxidation were given.

Methyl Ketone Formation from Mycelium

Although it is generally agreed (e.g. Berridge, Hiscox & Zielinska, 1953; Franke & Heinen, 1958) that a decline in methyl ketone formation occurs as spores germinate to mycelium, the innate ability of the mycelium to form ketones is still not completely proven. Knight and co-workers have published somewhat conflicting evidence. In 1955 Girolami & Knight obtained methyl ketones from the C₄ to C₉ fatty acids, and also considerable oxygen uptake, using mycelium of P.roqueforti. In 1958 Gehrig & Knight considered however that only spores and not mycelial cells were able to form methyl ketones from fatty acids. Later Haidle & Knight (1961) were unable to show for certain whether the mycelium of P.roqueforti had the ability to produce ketones or not as they were not able to obtain mycelium completely devoid of spores.

They found that during germination the ketone-producing activity decreased markedly but never disappeared completely. More recently Gehrig & Knight (1963) claimed that spores which had germinated were completely unable to take up oxygen when incubated with fatty acids, a surprising conclusion in view of the results of other workers who have investigated oxygen uptake during the oxidation of fatty acids by fungal mycelium.

Methyl Ketone Formation using Cell-free Extracts

Karrer & Haab (1948) were unable to isolate the β -keto acid decarboxylase but dried mycelial powders from various *Penicillia* were able to decarboxylate β -keto acids. They considered the decarboxylase to be highly unstable. More recently however, Franke, Platzek & Eichhorn (1961) isolated a constitutive and highly stable β -keto acid decarboxylase from *Aspergillus niger*. Optimum decarboxylase activity at C_{12} corresponded to one of the two optima of methyl ketone formation from fatty acids by washed mycelium (Franke & Heinen, 1958). There was however no corresponding decarboxylase activity for the other methyl ketone optimum at C_4 .

Haidle & Knight (1961) failed to obtain the methyl ketone forming system in the cell free state from spores. No activity was observed after sonic oscillation for prolonged periods in either the supernatant fraction, the broken spore wall fraction or a combination of both fractions.

TOXICITY OF FATTY ACIDS TOWARDS FUNGI

The marked effect of saturated fatty acids on the growth of fungi was first reported by Laxa (1902), who found that P.glaucum could grow in a medium containing 0.24% hexanoic acid but not in the same concentration of octanoic acid. Levine & Novak (1949) showed that the oxygen uptake of Blastomyces dermatitidis was stimulated by fatty acids up to C₈ acid but inhibited by higher acids. Webley & de Kock (1952) found that high concentrations (1% w/v) of the C₈ - C₁₂ acids completely inhibited oxygen uptake of Proactinomyces opacus at pH 7.0 but that low concentrations (0.0012M) were rapidly oxidised. Wyss, Ludwig & Joiner (1945) investigating the fungistatic action of fatty acids found that the optimum chain length varied with different organisms and with the solubility of the fatty acid in question. Thus for A.niger the optimum chain length was C₁₁ but was C₁₃ for the more sensitive T.interdigitale. Rigler & Greathouse (1940) found C₁₁ to be the acid most toxic to the growth of Phymatotrichum omnivorum.

Girolami & Knight (1955) showed that resting cell suspensions of P.roqueforti were capable of oxidising fatty acids in low substrate concentration (1.5 μ moles/ml). The acids became increasingly toxic as the carbon chain lengthened. Acids with more than 10 carbon atoms were oxidised at pH 7.3 only slowly, if at all, even at low substrate concentration. Rolinson (1954) found, on the other hand, that at pH 6.4 P.chrysogenum readily oxidised long chain fatty acids (C₁₆ - C₁₈) but that the intermediate C₈ - C₁₀ acids were toxic.

Thornton (1963) demonstrated that the $C_9 - C_{11}$ fatty acids showed greatest activity in inhibiting spore germination of Pithomyces chartarum. Vinze & Ghosh (1962) found that low concentrations (less than 2μ moles/ml) of C_8 and C_{10} acids stimulated mycelium of P. chrysogenum but were toxic at higher concentrations.

In general, considering the effect of homologous series, the toxicity on a molar basis rises with molecular weight but at some point the trend is reversed and higher members of the series are less toxic. The exact point of maximum toxicity depends upon the series and on the organism. The sharp decrease in toxicity with increased molecular weight is probably a reflection of insolubility of and micelle formation by the higher members of the series (Badger, 1946).

The toxicity of fatty acids, and of weak acids in general, has been found to be dependent on pH (Turner & Hanly, 1947; Beevers & Simon, 1949; Simon, 1950; Simon & Beevers, 1951), toxicity increasing as the concentration of undissociated molecules increases. When dissociated such substances may either be non-toxic or even result in an increase in oxygen uptake.

Webley & De Kock (1952) considered the toxic effect of the $C_8 - C_{12}$ acids on Proactinomyces opacus at pH 7.0 to be connected with their detergent properties. The biological activity of soaps arises from their power of concentrating at the biological surface, leading to an alteration in permeability and sometimes to lysis with the escape of cell contents. Similarly Nieman (1954) suggested that the toxic action of fatty acids is due to an accumulation of the active material in a peripheral layer or zone outside the semi-permeable

membrane. However it is now believed (Cochrane, 1959), although no experimental proof has been offered, that the primary inhibitory effect is not at this stage but later after some of the toxic substance has penetrated the protoplasm proper. Cochrane has proposed that toxicity is due to the differential accumulation of a toxic material by the organism, i.e., some materials which are perhaps equally toxic to all organisms may be accumulated selectively by a fungus.

The physiological importance of methyl ketone formation is not clear. Stokoe (1928) postulated that the abnormal oxidation of fatty acids to methyl ketones was a result of the poisonous or narcotic effect of the acid on the fungus. However the methyl ketones produced from toxic fatty acids are themselves inhibitory to the growth of fungi. Stokoe found that growth of P. palitans was inhibited by 0.2% 2-heptanone whereas, according to Girolami & Knight (1955), 0.1% 2-heptanone inhibited the growth of P. roqueforti and was a possible factor in preventing excessive fungal development in Blue cheese. Reduction of 2-heptanone to the less toxic 2-heptanol would be a further stage in detoxification of the acid. Franke, Platzek & Eichhorn (1962) considered that the ability of fungi to form methyl ketones might serve to act as a non-specific inhibitor against sensitive competitors for "Lebensraum" during germination.

Detoxification of fungicidal agents has been observed and may be a potential mechanism of resistance. Fawcett, Spencer & Wair (1955) demonstrated the importance of β -oxidation in the plant growth-regulating activity of the phenoxyalkyl carboxylic acids. Both β -oxidation of the fatty acid side chain and nuclear hydroxylation

in the 6-position accompanied detoxification of A.niger with higher members of the ω -(2-naphthoxy)-n-carboxylic acids (Byrde & Woodcock, 1956) and of Nocardia opaca with ω -phenyl substituted fatty acids (Webly, Duff & Farmer, 1955).

OXIDATION OF TRIGLYCERIDES TO METHYL KETONES BY FUNGI

In cheese the source of fatty acids, and thus possibly of the methyl ketones, is the triglycerides. The oxidation of triglycerides however by fungi has not been investigated in detail, particularly at the pH's to be found in Blue cheese. 24 hours after manufacture the pH of Blue cheese is approximately 4.6 rising to about pH 6.5 after 3-6 months, followed by a further fall to pH 5.5 after 9 months. (Coulter, 1938; Morris, Jezeski, Combs & Kuremoto, 1963).

Acklin (1925) found that P.glaucum oxidised trihexanoin in buffered solutions to give up to 10% of the theoretically possible amount of 2-pentanone. Thaler & Eisenlohr (1941) showed that the yields of methyl ketones (about 10-15%) from synthetic triglycerides containing $C_4 - C_{12}$ acids were greater than from the acids themselves. They found the formation of methyl ketones to be dependent on pH and followed a zig-zag course during prolonged incubation.

Mycelial lipases

The hydrolysis of the triglyceride is probably the

first step and this is known to be pH dependent. Thibodeau & Macy (1942) obtained maximum lipase activity of P.roqueforti between pH 5.3 and 7.5 and showed the optimum temperature for lipolysis to lie in the range 30 - 35°. These results have been confirmed generally by a large number of workers (Fodar & Chari, 1949; Parmelee & Nelson, 1949; Shipe, 1951; Wilson, Nelson & Wood, 1955; Imamura & Tsugo, 1953). Mycelium has been shown to possess both extracellular and intracellular lipases (Morris & Jezeski, 1953). Kirsch (1935) considered the lipases of P.oxalicum to be highly non-specific whereas Shipe (1951) found that C₄ and C₈ acids were released from tributyrin and tricaprylin in the ratio 3:1. Similarly Morris & Jezeski (1953) found that the activity of the lipases decreased as the molecular weight of the triglyceride increased.

Bacterial lipases are considered to be adaptive (Cutchins, Doetsch & Pelezar (1952) and similarly the lipolytic activity of P.roqueforti is greatly stimulated by adding fat to the growth medium (Nelson & Babel, 1946). Imamura & Tsugo (1953) however found that the lipolytic enzymes of P.roqueforti were not produced in any quantity until after 20 days incubation in the presence of butterfat.

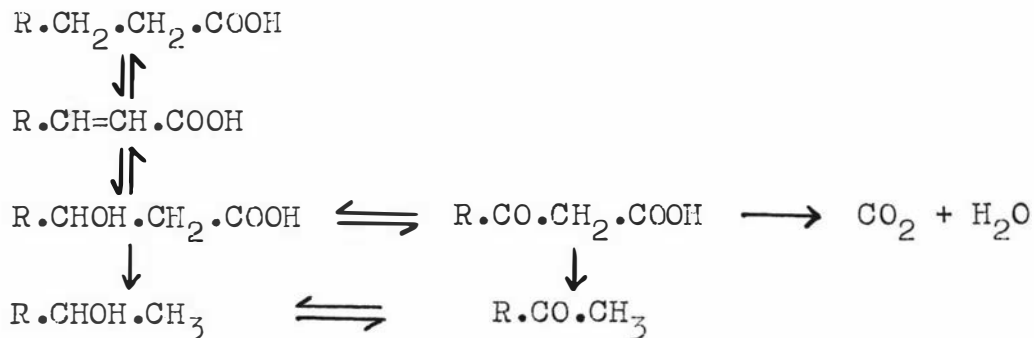
Spore lipases

The lipolytic activity of spores appears not to have been studied. Since spores in general and certain strains of *Aspergillus* and *Penicillia* in particular have high lipid content, spores might be expected to contain lipases. A feature of fungal lipids however is

the usually high proportion of free fatty acids (up to 88% of the total lipids, according to Raveux, 1948).

FORMATION OF METHYL CARBINOLS

In 1910 Haller & Lassieur detected in rancid coconut oil both methyl carbinols and methyl ketones and these Stokoe (1928) later showed were formed by oxidation of the oil by *Penicillia*. Neuberg & Lewite (1918) observed that yeasts gave up to a 10% yield of methyl carbinol when incubated with C₄ and C₅ methyl ketones. Acklin (1925) found carbinols in ripe bananas and cheeses as well as rancid fats and postulated that an intermediate methyl ketone was hydrogenated to the methyl carbinol. Stokoe (1928) on the other hand found that fungi oxidised methyl carbinols to methyl ketones far more readily than the latter were reduced to carbinols. He therefore postulated that the carbinols as well as the β-keto acids might be the precursors of the methyl ketones, basing his hypothesis on the assumption that decarboxylation of either the β-keto acid or the β-hydroxy acid could yield ketone, represented thus:



It is however generally assumed (e.g. Foster 1947; Wood, 1961) that methyl ketones are intermediates in methyl carbinol formation rather than vice versa, although evidence to support this view is limited. Thus in the fermentation of glucose by Clostridium butylicum to acetone and isopropanol, Osburn, Brown & Werkman, (1937) considered acetone to be the precursor because its addition enhanced the production of isopropanol. Similarly several groups of organisms (e.g. species of aerobacter, serratia, bacillus) produce acetoin and butanediol in glucose fermentations. De Moss, Bard, & Gunslaus, (1951) found that in Leuconostoc mesenteroides the reduction of acetoin is reversibly catalysed by butanediol dehydrogenase with the equilibrium in the direction of reduction :



They concluded that the balance between acetoin and 2,3-butanediol was determined by the amount of available hydrogen. Similarly Seitz, Sandine, Elliker & Day (1963) obtained evidence for a reversible 2,3-butanediol dehydrogenase in cell-free extracts of Streptococcus diacetylactis, the equilibrium probably favouring the reduction of acetoin to 2,3-butanediol.

Jackson & Hussong (1958) found that mycelium of P. roqueforti reduced 2-heptanone to 2-heptanol and vice versa. Unlike Stokoe (1928), however, they found the reduction to carbinol to proceed more readily than the oxidation to ketone. Similar results were obtained by Franke, Platzeck & Eichhorn (1962) who found that when Aspergillus niger oxidised C₁₂ acid, the C₁₁ methyl

ketone concentration built up to a maximum but was further metabolised on longer incubation. The amount of C₁₁ methyl carbinol found at the end of the experiment corresponded to the maximum amount of C₁₁ methyl ketone obtained. However, they also confirmed the earlier findings of Stokoe (1928) and Jackson & Hussong (1958) that mycelium also oxidises secondary alcohols to methyl ketones. Bassett & Harper (1956) isolated the C₈ and C₁₀ β-keto acids from Blue cheese which suggests that these are more likely precursors of methyl ketones than secondary alcohols. Jackson & Hussong (1958) also established that methyl ketones appear much sooner and in larger amounts than the secondary alcohols during the ripening of Blue cheese.

There is to date not enough evidence to justify deciding between the two pathways originally postulated by Stokoe (1928) although it would seem more likely that carbinols are reduction products of the ketones.

AIMS OF THE PRESENT INVESTIGATION

Since attention has recently been centred on the contribution of methyl ketones to the characteristic flavour of cheeses it was considered important to examine more precisely their formation in cheese, with particular reference to the possible mechanisms by which they, and related compounds such as methyl carbinols, could be formed.

Wong, Patton & Forss (1958) considered β -keto acids to be the precursors of methyl ketones found in steam distillates of evaporated milk and put forward, without experimental evidence, two alternative hypotheses to account for their origin:

- (1) as intermediates in the synthesis of fatty acids from acetate in the mammary gland
- (2) as degradation products of fatty acids by β -oxidation.

The first hypothesis implies that methyl ketones could be formed as artifacts during steam distillation of products containing milk fat. This possibility was considered by Harvey & Walker (1960) but they concluded that the methyl ketones obtained in their work were not formed as artifacts during the distillation but were present as such in Cheddar cheese. The point did not, however, seem to be clearly established and one object of the present investigation was to determine whether, and if so to what extent, methyl ketones were formed as artifacts during distillation.

As the work developed it became clear that bound precursors in milk fat could account for most, if not all,

the methyl ketones to be found in Cheddar cheese. Studies with radioactive milk fat allowed further investigation of the origin of methyl ketones and their precursors.

The second hypothesis of Wong et al (1958) suggests that the precursors of the methyl ketones are, in the first instance, triglycerides and fatty acids and that the agents of β -oxidation are microbiological. The hypothesis, as it applied to Cheddar cheese, was tested directly by adding synthetic triglycerides, containing fatty acids that are present in trace amounts only in milk fat, and determining the extent of methyl ketone formation.

For reasons outlined in the Introduction it was considered desirable to make a detailed study of the oxidation of fatty acids and triglycerides by the fungus Penicillium roqueforti, chosen as a general representative of lipolytic organisms that might be of importance in producing Cheddar flavour, and on account of its known ability to oxidise triglycerides and fatty acids to methyl ketones. Reports in the literature of β -oxidation by fungi were fragmentary and to some extent conflicting and it was hoped that a detailed study of the one organism might clarify the situation. No complete investigation of the oxidation of the homologous series of fatty acids over a wide range of pH has been reported for resting cells of fungi. In particular it was necessary to ascertain the relative abilities of spores and mycelium to form methyl ketones. It became evident that the toxicity of fatty acids towards fungi and the formation of methyl ketones were inter-related and the scope of the investigation was widened in an

attempt to understand the mechanism of toxicity.

This thesis has for convenience been presented in three parts. Part I contains the investigation into the formation of methyl ketones in steam distillates from Cheddar cheese and milk fat. Part II is an account of the formation of methyl ketones from cheeses to which synthetic triglycerides containing fatty acids, normally present in trace amounts only in milk fat, had been added. Part III is a study of the oxidation of fatty acids and triglycerides by the fungus Penicillium roqueforti.

Part I. THE ORIGIN OF METHYL KETONES
IN CHEDDAR CHEESE AND IN MILK FAT

MATERIALS

The cheeses used were manufactured in the Dairy Research Institute (N.Z.) factory by normal New Zealand commercial methods. They were kept at 13° for 2 weeks and then at 7° for the remainder of the ripening period.

Cheese-fat was obtained by grating the cheese, placing it in centrifuge tubes and melting it in a 60° waterbath. The melted cheese was centrifuged for 5 min. at 2,000 r.p.m. and the cheese-oil then poured off. Milk fat was prepared by melting fresh butter in centrifuge tubes and centrifuging as for cheese-oil.

Radioactive milk fat. A lactating dairy cow, after milking, was injected in the jugular vein with 5.0 mc sodium [carboxy-¹⁴C] acetate. The cow was milked 3, 9 and 22 hours after injection, the milk yields and activities of the milk fats being shown in Table 1. The milk fats were stored at - 10° until required for analysis.

Table I. Milk yield and milk fat radioactivity after the injection of carboxy-¹⁴C acetate

Milk Sample	Time after injection (hr.)	Milk yield (oz)	Activity of milk fat (uc/g atom C)
1	3	24	2.27
2	9	88	12.00
3	22	88	4.92

Carbonyl free solvents. Solvents were rendered carbonyl-free by passing them down a celite column impregnated with 2,4-DNP hydrazine (throughout this thesis 2,4-DNP

denotes 2,4-Dinitrophenyl), phosphoric acid and water, according to the method of Scwartz & Parks (1961). After passage through the column the solvents were distilled at atmospheric pressure.

METHODS

Isolation of radioactive fatty acids. The milk fat was saponified by the method of Henry & Kon (1942) and the soaps converted into free fatty acids with 40% (v/v) H_2SO_4 (10% excess) and steam distilled. The steam-volatile fatty acids were neutralized with KOH and evaporated to dryness. The non-volatile fatty acids were extracted with diethyl ether, washed with water until neutral to Congo red and dried "in vacuo" on a water bath. The acids were then separated into "solids" and "liquids" by crystallization three times from 15 vol. of acetone at -38° . The "solids" and "liquids" respectively were converted into methyl esters and fractionated in a spiral column (Shorland, 1952).

Carbon -14 counting. Counts were usually made at 2 amplitudes (normally 80 v and 150 v) since the ratio of counts at the 2 amplitudes for each sample should remain the same. Only samples giving considerably more than 3 standard deviations over the background count were reported as being radioactive, the standard deviation being the value of the square root of the total number of counts made at any one time.

Determination of specific activities

(a) Radioactive steam volatile fatty acids. These were resolved into their components by gas liquid chromatography (Aerograph Autoprep A.700, Wilkins Instrument Co.) using a column packing of 60% (w/w) diethylene glycol adipate and 2% phosphoric acid (85% H_3PO_4) on 30 - 80 mesh "Celite 545" (Metcalf, 1960). The acids were washed from the Autoprep collecting bottles with 4 ml toluene into a counting vial. Two aliquots of 1 ml were transferred to 100 ml round bottomed flasks, 1 ml of N/10 alcoholic KOH added to each and the excess alkali back titrated with N/100 sulphuric acid, using phenolphthalein as an indicator. 1ml scintillation fluid (0.05% 2,5 diphenyloxazole + 0.03% 1,4-di-[2 - (5-phenyloxazolyl)] - benzene in toluene) was added to the remaining 2 ml and counted at room temperature in a liquid scintillation system (NE 8301., Nuclear Enterprises (G.B.), Ltd., Edinburgh).

(b) Methyl esters of radioactive fatty acids. The "liquid esters" and "solid esters" were resolved into their components using a column packing of 60% (w/w) diethylene glycol adipate in the Autoprep. The esters were washed from the collecting bottles with 4 ml petroleum ether (40° - 60° fraction) into counting vials. A suitable aliquot was removed from each vial, the petroleum ether carefully evaporated off under reduced pressure at room temperature, and the concentration of the ester determined by the method of Morgan & Kingsbury (1959). This is based on the formation of hydroxamic acids which react with ferric chloride to form a coloured

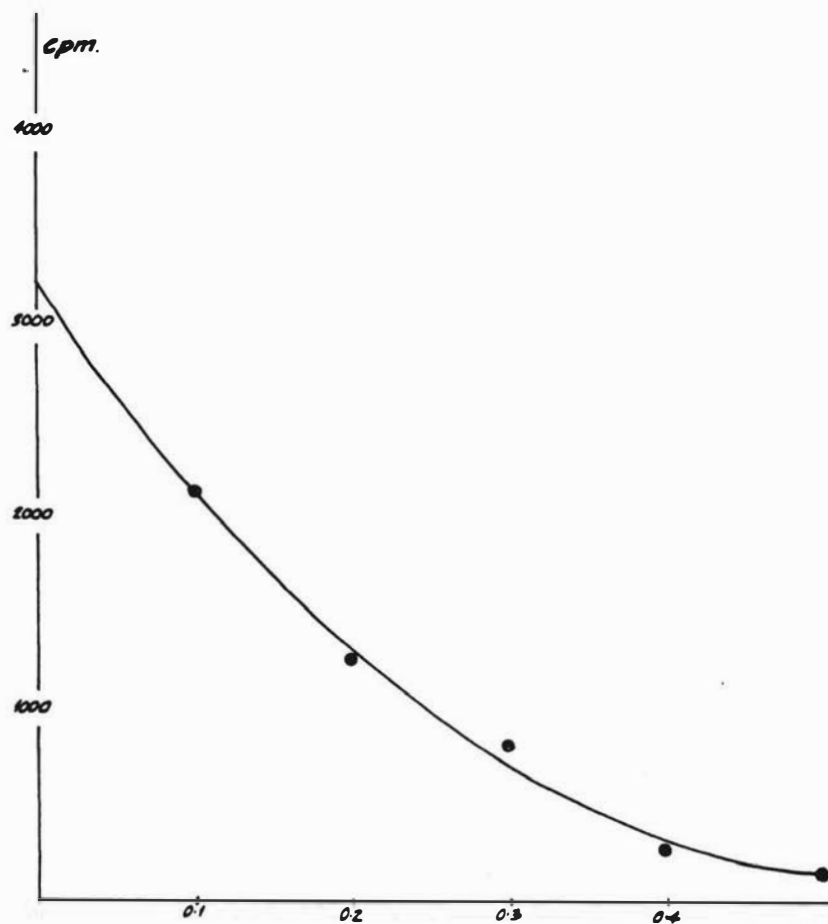
complex having an absorption maximum at 515 m μ . A very small correction had to be made for a trace of adipate that leaked off the column during the collection of each ester.

The petroleum ether was carefully removed at room temperature under reduced pressure, 3 ml of scintillation fluid added and the vials counted as above.

(c) 2,4-DNP hydrazones of radioactive methyl ketones. Suitable aliquots of each of the 2,4-DNP hydrazones (less than 0.2 mg) were dissolved in 3 ml scintillation fluid and counted as above.

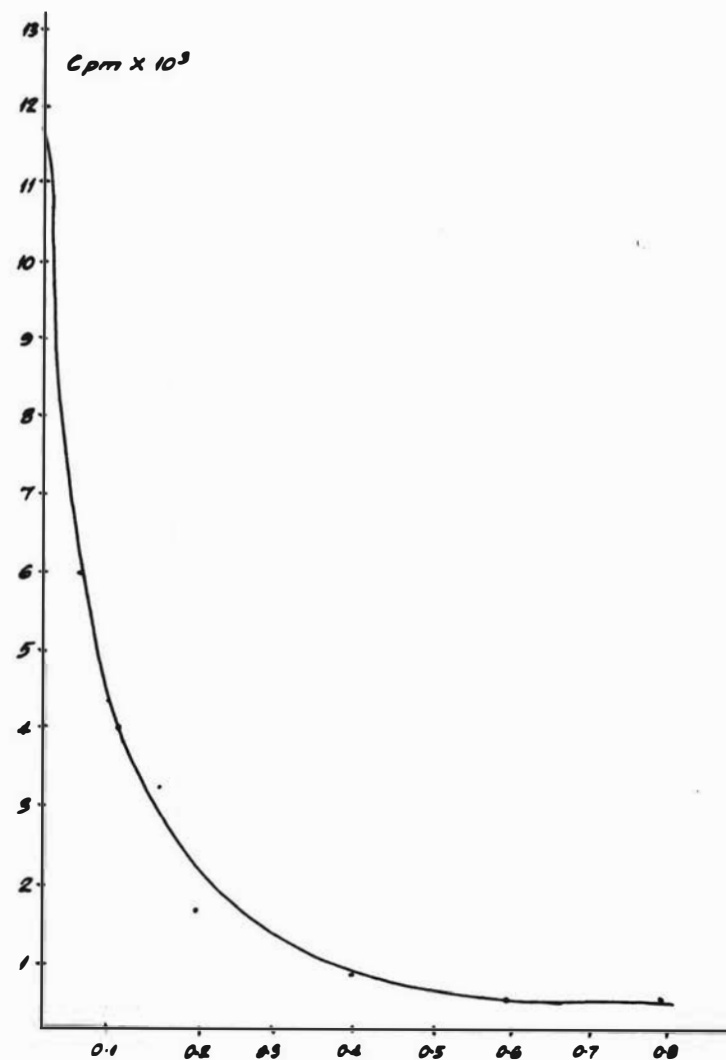
Quenching corrections. These were established by the use of ^{14}C -n-hexadecane as an internal standard. Quenching curves were prepared for the 2,4-DNP hydrazones of the methyl ketones from C_3 to C_{15} , that for 2-heptanone (Fig. 1) being typical for the quenching of a high initial count and that for 2-undecanone (Fig. 2) for a lower count. In all cases the quenching of radioactivity was found to be proportional to the amount of 2,4-DNP hydrazone added, up to a maximum of 0.2 mg. The corrections necessary to compensate for quenching were calculated from the quenching curves and, also as a check, by adding a known aliquot of the standard ^{14}C -n-hexadecane dissolved in toluene to the vial containing the radioactive 2,4 DNP hydrazone.

In an attempt to overcome quenching of the hydrazones, the less volatile methyl ketones from C_7 to C_{15} were regenerated from the corresponding 2,4 DNP hydrazones with laevulinic acid (Keeney, 1957). Much less quenching was encountered and the correction factors



Mgs 2,4-DNP hydrazone

Fig.2. The quenching effect on ^{14}C - n hexadecane of the 2,4-DNP hydrazone of 2-undecanone.



Mgs 2,4-DNP hydrazone

Fig.1. The quenching effect on ^{14}C - n hexadecane of the 2,4-DNP hydrazone of 2-heptanone.

needed, as determined by the use of ^{14}C -n-hexadecane, were correspondingly smaller. Thus the correction factor determined after regeneration of 2-undecanone was 1.15 compared to the factor 1.45 needed when the radioactivity of its 2,4 DNP hydrazone was determined directly. Since the above method was not suitable for the regeneration of acetone and 2-pentanone, specific activities for methyl ketones recorded in these investigations are those calculated from radioactivity measurements on the 2,4 DNP hydrazones directly.

Corrections necessary for quenching by the steam volatile fatty acids and methyl esters were very small, never greater than 1.1.

Collection of methyl ketones

Steam distillation at atmospheric pressure. For experiments with cheeses 1 Kg portions (except in preliminary experiments when 500 g were used) were coarsely grated, covered with a litre of distilled water and steam distilled at atmospheric pressure. Preliminary experiments showed that the yield of carbonyls from a cheese was dependent upon the rate of distillation and in an attempt to standardize the procedure it was therefore arranged that the rate of distillation was uniform. The distillate was led directly into 100 ml of 2,4 DNP hydrazine in 2N-HCl and the distillate-reagent mixture was then held at room temperature for 24 hours. The 2,4 DNP hydrazones were extracted with carbonyl-free chloroform and the

extract evaporated to dryness. The hydrazones were taken up again in the minimum quantity of petroleum ether or hexane for subsequent column chromatography.

Methyl ketones were similarly isolated as 2,4 DNP hydrazones from steam distillates of 400 g cheese fat and milk fat and of 1 kg cream, the quantities of fat thus being approximately the same in all the materials used. For experiments with radioactive milk fat 20 g only of the milk fat from each of the three milkings were steam distilled.

Steam distillation under reduced pressure. This was carried out as described above except that the pressure throughout the apparatus was reduced to keep the temperature of distillation at approximately 40°.

Continuous stripping of volatiles from cheese extract with nitrogen at room temperature. 1 Kg of the cheese was blended with 1500 ml of carbonyl-free benzene. The benzene filtrate was placed in a flask in a closed circuit including a trapping solution of 2,4-DNP hydrazine and a sealed pump (Reciprotor). The apparatus was flushed with nitrogen to displace air and the system was sealed. The nitrogen was circulated continuously for 2 weeks by means of the pump.

Purification of radioactive methyl ketones

The 2,4-DNP hydrazones were subjected to a series of chromatographic procedures to remove neutral fat, fatty acids, alkanals and alkenals, which as trace contaminants

would contribute radioactivity to the 2,4-DNP hydrazones of the methyl ketones.

Removal of neutral fat. A hexane solution of the 2,4-DNP hydrazones of the methyl ketones, containing also the 2,4-DNP hydrazones of contaminating alkanals and alkenals, was added to a column of activated magnesia (Seasorb 43) : celite 545 (1 : 1). Traces of fat were first eluted with hexane (Schwartz, Haller & Keeney, 1963), the 2,4-DNP hydrazones then being eluted with nitromethane : chloroform (1 : 3) and evaporated to dryness.

Removal of fatty acids. The dry residue from the magnesia : celite column dissolved in a minimum of chloroform was added to an alumina column (2 g packed in about 4 ml. chloroform). Collection of the effluent was begun immediately, chloroform being added until the effluent became colourless. Any contaminant fatty acids remained on the column.

Removal of 2,4-DNP hydrazones of alkanals and alkenals.

Naturally occurring alkanals and alkenals have been reported in milk fat (Parks, Keeney & Schwartz, 1961) and were isolated in steam distillates. In the investigation with radioactive milk fat small amounts of alk-2-4-dienals were also detected, indicating chemical deterioration during storage, although precautions were taken to prevent autoxidation. The concentration of carbonyls other than methyl ketones varied between 5 and 10% of the total carbonyls obtained, but were not radioactive. No methyl ketones have however been reported as products of the autoxidation of fats.

To separate the 2,4 DNP hydrazones of the methyl ketones from those of other carbonyls the dry residue from the alumina column was taken up in 15% chloroform in hexane, added to a column of magnesia 2665 : celite (1 : 1) and the 2,4-DNP hydrazones of the methyl ketones eluted with more 15% chloroform in hexane, according to the procedure of Schwartz, Parks & Keeney (1962).

Chromatographic separation of 2,4 DNP hydrazones. Initial separation of the 2,4 DNP hydrazones was carried out in preliminary experiments by the method of Monty (1958) but the column partition chromatography method of Day et al. (1960) was subsequently found to be more suitable and was employed for all later work.

Identification of 2,4 DNP hydrazones

Tentative identification of the 2,4 DNP hydrazones was accomplished by comparing retention volumes of the unknown derivatives with those reported by Monty (1958) and Day et al. (1960) for known derivatives. The fractions from the column were collected separately and evaporated to dryness for subsequent paper chromatographic analysis. The method of Huelin (1952) was used to identify carbonyls up to C₇ and that of Klein & de Jong (1956) for carbonyls from C₅ to C₁₅. The method of Lynn, Steele & Staple (1956) was used to confirm the identification of the intermediate carbonyls from C₄ to C₉.

Further identification of the 2,4 DNP hydrazones was possible by measurement of their light absorption maxima in ethanol and particularly by fading studies of their spectra in alkaline ethanolic solution according to Jones, Holmes & Seligman (1956). Particular attention was paid to the subsidiary peak in the 520 μ region which disappeared with saturated aliphatic aldehydes but persisted with saturated aliphatic ketones. For this purpose a suitable aliquot of the 2,4 DNP hydrazone of an unknown carbonyl from the column, or spot eluted from a paper chromatogram, was mixed with 0.25 N ethanolic sodium hydroxide and the absorption spectrum in the 500 to 540 μ region was measured immediately and again after an interval of 90 min.

By these methods it was possible not only to identify the methyl ketones present but also to obtain quantitative comparisons of their concentration. In preliminary experiments, methyl ketones not normally found in cheese distillates were added in known concentrations to cheeses of various ages and to milk fat, and recovery experiments carried out as reported later. The 2,4-DNP hydrazones of the methyl ketones found in the steam distillates could thus be compared directly against markers of known concentration.

For more accurate quantitative estimations, known weights of the 2,4-DNP hydrazones of each of the methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} were dissolved in alcohol and standard curves prepared relating absorption at 362 μ to original concentration of methyl ketones. Having identified a methyl ketone it was thus possible to obtain a measure of the quantity present in the distillate. However, as

pointed out by Wong, Patton & Forss (1958), a difficulty in the estimation of methyl ketones in this type of investigation is the non-quantitative nature of the reaction of carbonyls with the 2,4-DNP hydrazine reagent. It was not found possible to standardize completely the procedure of steam distillation at atmospheric pressure and reproducibility for the same sample of cheese or of milk fat was found to be about $\pm 5\%$ for C₇ and C₉ and $\pm 15\%$ for the other methyl ketones. Nevertheless 2,4-DNP hydrazine at present continues to be the most satisfactory reagent available for the isolation and identification of carbonyl compounds.

EXPERIMENTAL

Methyl ketone patterns found in steam distillates of cheeses manufactured under controlled bacteriological conditions in "aseptic" vats.

Perry & McGillivray (1964) designed an apparatus that successfully excluded bacteria from the vat during cheesemaking. This enabled cheeses to be made that contained no bacteria or only starter bacteria, micrococci, lactobacilli (or combinations of these organisms) and allowed the overall importance of these bacteria in cheese ripening to be assessed. None of these cheeses made "aseptically" developed typical

Cheddar flavour although control cheeses, manufactured under the same conditions but with the lid of the vat off, were normal in flavour.

After bacteriological examination the cheeses (30 "aseptic" and 8 controls) were chemically analysed. Steam distillations of cheese slurries were carried out according to the method described by Harvey & Walker (1960), except that 1 litre of distillate was collected. Despite the wide variations in flavour found in the cheeses and the very different bacterial populations present, the pattern of methyl ketone formation did not differ significantly. 2-alkanones with odd numbers of carbon atoms from C₃ to C₁₅ were identified in approximately the same total and relative concentrations in each of the cheeses irrespective of flavour and bacterial flora. In general the relative concentrations were 2-heptanone > acetone > 2-pentanone > 2-nonanone > 2-undecanone > 2-tridecanone > 2-pentadecanone.

Since approximately the same concentrations of methyl ketones were found in the steam distillates from cheese with absolutely no Cheddar flavour as from control cheese with typically characteristic flavour, it seemed highly probable that either there was no correlation between flavour and methyl ketone concentration or that the methyl ketones were being formed as artifacts during the steam distillation.

A detailed investigation was therefore undertaken in an attempt to determine whether, and if so to what extent, methyl ketones were formed as artifacts during distillation of Cheddar cheese.

Isolation of methyl ketones from Cheddar cheese and milk fat by steam distillation at atmospheric pressure

A range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} was obtained from cheeses of all ages with no indication of significant differences in methyl ketone pattern with increasing maturity of the cheeses (Table 2). It was, however, noted that considerably larger quantities of methyl ketones could be obtained by continuing the distillation for longer periods and that the concentration of methyl ketones in successive 200 ml fractions of distillate did not decrease appreciably until at least 1000 ml of distillate had been collected.

In contrast to this persistence of methyl ketones with odd numbers of carbon atoms, recovery trials in which known amounts of 2-hexanone, 2-octanone and 2-decanone, even carbon numbered methyl ketones present normally only in trace amounts in cheeses, were added to water, milk fat or cheese slurries resulted in the anticipated decreasing concentrations of these substances in successive 200 ml fractions of distillate. Traces of these ketones were present in the fourth 200 ml fraction collected but they were not detected in subsequent distillates. Recoveries were almost quantitative from water or milk fat and approximately 85% from cheese slurries.

These findings suggested that part at least of the methyl ketones obtainable from cheese by steam distillation at atmospheric pressure were not present as such in the cheese but were being formed as artifacts during the distillation. Any methyl ketones present in the

Table 2. Variation in concentration of methyl ketones found in 1000 ml steam distillates at atmospheric pressure from cheese, cheese-oil, milk fat and cream (fat content of each sample approximately 400 g)

	Age	Methyl ketones (concentration in mg)							
		C ₃	C ₄	C ₅	C ₇	C ₉	C ₁₁	C ₁₃	C ₁₅
Cheese E	1 day	1.9	-	1.7	4.0	1.1	0.3	0.3	0.1
Cheese F	1 month	1.4	0.1	1.1	3.7	1.2	0.5	0.2	0.2
Oil from Cheese F	1 "	1.4	-	0.3	3.7	1.3	0.5	0.2	0.1
Cheese G	9 months	1.9	0.6	1.1	4.1	1.3	0.6	0.3	0.2
Cheese H	9 "	1.5	0.9	1.8	3.4	1.2	0.5	0.3	0.2
Oil from Cheese H	9 "	1.0	0.3	0.7	3.0	0.8	0.3	0.3	0.3
Cheese D	13 months	1.4	0.3	1.1	3.3	1.5	0.3	0.1	0.1
Milk fat	1 day	1.9	0.1	1.2	5.4	1.7	0.5	0.3	0.1
Cream	Fresh	1.4	0.1	0.6	3.4	1.3	0.5	0.2	0.2

free state in cheese, at least up to C_{11} , would be expected to distil over in the first 1000 ml of distillate collected and experiments were therefore carried out in which cheeses of various ages from 1 day to 13 months were distilled, 1 litre of distillate being collected and analysed for methyl ketones. As the results shown in Table 2 indicate, the same range of 2-alkanones with odd numbers of carbon atoms from C_3 to C_{15} were identified in approximately the same total and relative concentrations in each of the cheeses irrespective of age. In addition to the 5 cheeses reported in Table 2, nine other cheeses aged between 1 day and 9 months were examined and showed little variation from this general pattern. There were however, significantly higher concentrations of 2-butanone found in mature cheese than in young cheese. That the methyl ketones, or their precursors, were associated with the fat fraction of the cheese was shown by "oiling off" samples of cheese and steam distilling the cheese-fat obtained. These cheese-fats yielded the same range and relative concentrations of methyl ketones as did the cheeses from which they had been prepared (Table 2). The association of methyl ketones or their precursors with the fat phase was further demonstrated by the steam distillation at atmospheric pressure of fresh cream (40% fat) and also of fresh milk fat obtained by oiling off the butter from similar cream. As Table 2 shows, the same range and relative concentrations of methyl ketones from C_3 to C_{15} were obtained as before. Furthermore successive 200 ml fractions of distillate from cream and milk fat showed the same pattern of methyl ketone persistence as did

comparable fractions from cheeses indicating that the same type of precursor breakdown was probably occurring in cream and milk fat as in the cheeses.

Identification of methyl ketones with an even number of carbon atoms

Trace amounts of methyl ketones with an even number of carbon atoms from C_4 to C_{16} were also detected in the steam distillates of both cheese and milk fat. Only 2-hexanone and 2-octanone were isolated in significant amounts and these were less than 2% of the concentration of 2-heptanone. Traces only of 2-butanone were found in distillates from milk fat and young cheese but comparatively large concentrations were found in some mature cheese (Table 2).

Exhaustive steam distillation of cheese and milk fat

As shown in Tables 3 and 4, exhaustive steam distillation of cheese or milk fat resulted in a slow decrease in concentration of the C_3 to C_{11} methyl ketones in successive litre fractions of distillate. Individual methyl ketones, however, varied markedly in their persistence and the close similarity between the two tables afforded further evidence of the identity of the precursors of the methyl ketones in milk fat and in cheese. Thus the 10 l of distillate collected

contained all the 2-heptanone and 2-nonanone, present as such or formed as artifacts during heat treatment, and most of the 2-pentanone and 2-undecanone, but recovery of acetone, 2-tridecanone and 2-pentadecanone was still incomplete. Further experiments were therefore carried out in which smaller quantities (10 g) of milk fat were exhaustively distilled until no further methyl ketones could be detected in 2 l fractions of distillate. In using these smaller quantities of milk fat the dimensions of the apparatus were correspondingly reduced to ensure effective steam distillation. It was found necessary to collect 10 l of distillate. Total concentrations of methyl ketones so obtained from two separate samples of milk fat are shown in Table 5.

Isolation of methyl ketones from cheese and milk fat by steam distillation under reduced pressure

The preceding experiments indicated that extraction procedures involving high temperatures were of doubtful value for the estimation of preformed methyl ketones in dairy products containing milk-fat. Alternative methods of extraction involving less drastic heat treatment were therefore investigated.

Experiments were carried out in which cheeses, cream and milk fat were steam distilled at 40°C under reduced pressure. Under these conditions the same range of methyl ketones with odd numbers of carbon atoms from C₃ to C₁₅ was obtained but the yields were very considerably

Table 3. Variation in concentration of methyl ketones found in successive 1000 ml fractions of steam distillate at atmospheric pressure of 1 Kg of a 7 month old Cheddar cheese

Fraction	Methyl ketones (concentration, mg)						
	C ₃	C ₅	C ₇	C ₉	C ₁₁	C ₁₃	C ₁₅
1	2.3	2.1	4.8	2.0	1.4	0.3	0.2
2	2.5	1.0	2.1	1.2	1.4	0.4	0.3
3	2.0	0.5	0.6	0.7	1.3	0.6	0.3
4	2.1	0.4	0.4	0.4	0.6	0.6	0.5
5	1.3	Tr	Tr	0.1	0.4	0.6	0.6
6	1.1	Tr	-	Tr	0.2	0.5	0.5
7	1.0	-	-	-	Tr	0.3	0.5
8	0.6	-	-	-	Tr	0.3	0.4
9	0.9	-	-	-	Tr	0.3	0.4
10	0.5	-	-	-	Tr	0.3	0.5

Tr = Trace, i.e. less than 0.1 mg

Table 4. Variation in concentration of methyl ketones found in successive 1000 ml fraction of steam distillate at atmospheric pressure of 400g fresh butter-oil

Fraction	Methyl ketones (concentration in mg)						
	C ₃	C ₅	C ₇	C ₉	C ₁₁	C ₁₃	C ₁₅
1	2.8	1.9	5.5	1.8	1.1	0.3	0.1
2	2.1	1.0	2.4	1.8	1.9	0.6	0.2
3	2.0	1.2	1.1	1.2	0.9	0.6	0.6
4	1.2	0.5	0.5	1.0	1.1	0.8	0.5
5	1.4	0.4	0.5	0.8	0.9	0.8	0.5
6	1.0	0.5	0.3	0.8	0.8	0.7	0.8
7	0.6	0.3	Tr	0.5	0.6	0.9	0.8
8	0.6	Tr	Tr	Tr	0.2	0.4	0.9
9	0.7	Tr	-	-	Tr	0.3	0.8
10	0.5	Tr	-	-	Tr	0.4	0.6

Tr = trace, i.e. less than 0.1 mg

Table 5. Concentration of methyl ketones (p.p.m.)
obtained from milk fat samples by exhaustive
steam distillation at atmospheric pressure

Sample	100°		Average	180° (*)
	1	2		
C ₃	45	39	42	-
C ₅	16	10	13	-
C ₇	28	22	25	12
C ₉	20	12	16	9
C ₁₁	15	13	14	13
C ₁₃	28	20	24	22
C ₁₅	51	41	46	42

* As reported by Bolding & Taylor (1962)

lower than from corresponding samples steam distilled at atmospheric pressure. At 40°, however, there was still evidence of the formation of methyl ketones as artifacts. Thus in recovery trials in which 2 mg 2-octanone were added to milk fat and cheese, it was found that all the recoverable 2-octanone came over in the first three 250 ml fractions of distillate. In contrast acetone, 2-pentanone and 2-heptanone were present in the fourth 250 ml fraction in approximately the same concentrations as in each of the first three 250 ml fraction indicating, as is the case of steam distillation at 100°, that they were being formed as artifacts during distillation. However, the concentrations of methyl ketones found in low temperature distillates from mature cheeses were greater than those obtained under the same conditions from young cheeses, cream or milk fat, particularly the methyl ketones from C₇ to C₁₅. Since the quantities of fat were approximately the same in all samples it is probable that not all of the methyl ketones obtained from mature cheese were formed as artifacts during distillation and that some at least were present as such in the cheeses.

Continuous stripping of volatiles from cheese extracts with nitrogen at room temperature

Further evidence indicating that free methyl ketones do exist in mature cheese was provided by passing nitrogen continuously through benzene extracts of the cheese for two weeks at room temperature. The homologous series

of methyl ketones with odd numbers of carbon atoms from C_3 to C_9 was found in a 6 month old cheese and from C_3 to C_{11} in a 9 month old cheese. 2-butanone also was found in both cheeses and 2-hexanone in the 9 month old cheese. The amounts of methyl ketones obtained were very small. They were of the order of 5% of those obtained by steam distillation at atmospheric pressure. When, however, a further Kg of the same 9 month old cheese containing 2 mg of added 2-heptanone was extracted with benzene approximately 40% of the 2-heptanone was recovered by passage of nitrogen continuously through the apparatus for 2 weeks. This method of extraction was thus shown to be capable of stripping free methyl ketones from the benzene extract of cheese when they were present. This was further evidence that a considerable portion of the methyl ketones found in distillates at atmospheric pressure are artifacts.

The low yields of methyl ketones isolated by this method and also by steam distillation under reduced pressure were not explained until much later in the investigation when the high volatility of the methyl ketones in aqueous solution and the consequent instability of the 2,4-DNP hydrazones became apparent (Part 3, page 105).

Isolation of carbonyls from coconut oil by steam distillation at atmospheric pressure

To determine if there were any connection between the relatively large proportions of short chain (C_4 to C_{14}) fatty acids and the presence of β -keto acids in milk fat, it was of interest to see if methyl ketones could be detected in steam distillates of a lipid such as coconut oil, which also contains a high proportion of short chain fatty acids. The triglyceride composition of coconut endosperm is characterised by a high proportion of C_8 - C_{16} saturated fatty acids (91% by weight) of which C_{12} acid constitutes 51% by weight (Hilditch, 1956).

10 g fresh dried coconut oil were steam distilled exhaustively, the carbonyls in the distillate being isolated as the 2,4-DNP hydrazones which were extracted and separated on a celite/nitromethane column. The identity of the various 2,4-DNP hydrazones was determined by their separation into classes (alkanals, 2-alkanones, alk-2-enals) by thin layer chromatography (Urbach, 1963) and further characterised by paper chromatography. Very small amounts (less than 5 p.p.m.) of C_9 and C_{14} n-alkanals were detected. No methyl ketones except acetone (25 p.p.m.) were found suggesting that any C_6 to C_{16} β -keto acids incorporated into the triglycerides of coconut oil are presumably reduced in situ and that the presence of β -keto acids in the triglycerides of milk fat is a result of their rapid removal from the site of synthesis.

Comparison of radioactivities in methyl ketones and fatty acids from milk fat after injection of carboxy-¹⁴C acetate

The evidence up to this point in the investigation suggested a possible relationship between methyl ketone precursors and fatty acid synthesis. An examination of fatty acids from the milk fat of a cow which had been injected with [carboxy-¹⁴C] acetate and of methyl ketones obtained by steam distillation of the same radioactive fat allowed a direct comparison of the two classes of compounds to be made. Popják, French, Hunter & Martin (1951) had analysed the fatty acids obtained from milk fat of a goat after intravenous injection of 5 mc [carboxy-¹⁴C] acetate but no similar experiment had been conducted on a cow. It seemed possible that different results might be obtained since the fatty acid composition of goat milk fat is very different to that of the cow, the C₄ - C₁₂ acids in goat milk fat representing about 36 moles % of total acids and only about 22 moles % for the cow. The relative amounts of the individual acids are also different, C₁₀ and C₄ acids representing 11.7 and 6.6 moles % of total acids in goat milk fat (Hilditch, 1956), and 1.4 and 9.1 moles % in cow milk fat (Hawke, 1957).

Table 6 shows the yields of methyl ketones obtained by steam distillation of radioactive fat from each of the three milkings. Only the fat from the second milking was steam distilled exhaustively but no significant difference was apparent except for the greater yield of C₁₅ methyl ketone. No methyl ketones with carbon number greater than C₁₅ were detected in

Table 6. Yields of methyl ketones (p.p.m.) obtained by steam distillation at 100° of milk fats from a cow milked 3, 9 and 22 hr. after intravenous injection of carboxy-¹⁴C acetate

Methyl ketone	1st milking [*] p.p.m.	2nd milking ^{**} p.p.m.	3rd milking [*] p.p.m.
C ₁₅	20	38	21
C ₁₃	17	17	18
C ₁₁	10	14	14
C ₉	11	10	7
C ₇	14	14	7
C ₅	6	9	10
C ₃	18	20	16

* 6 litres distillate collected

** 10 " " "

any of the distillates. The atypical yields of C₇ and C₉ methyl ketones obtained from the fat of the third milking can possibly be attributed to the fact that 1 litre of blood was drawn from the cow between the second and third milking. This would probably affect the milk fat composition (Munford, 1964).

¹⁴C labelling patterns of methyl ketones

Figures 3 - 5 show the relationship between the specific activities of the individual methyl ketones obtained from milk fats from the three milkings and the chain length of the methyl ketones. The methyl ketones obtained from the fat from the first milking had relatively low specific activities decreasing from 2.6 $\mu\text{c/g}$ atom C for 2-pentanone to 0.5 $\mu\text{c/g}$ atom C for 2-pentadecanone. The low activities detected in the first milking, between 3 and 10 counts only above background, necessitated long counting times. The specific activities of the methyl ketones obtained from the fat of the second milkings were considerably higher than those from the first milking, a peak value of 22.5 $\mu\text{c/g}$ atom C being reached for 2-undecanone. At the third milking, 22 hours after injection of the acetate, the specific activity of all the methyl ketones had decreased from the values of the second milking, the highest values being found in the C₁₁, C₁₃ and C₁₅ methyl ketones. Peak activity was again obtained at C₁₁ (12.5 $\mu\text{c/g}$ atom C).

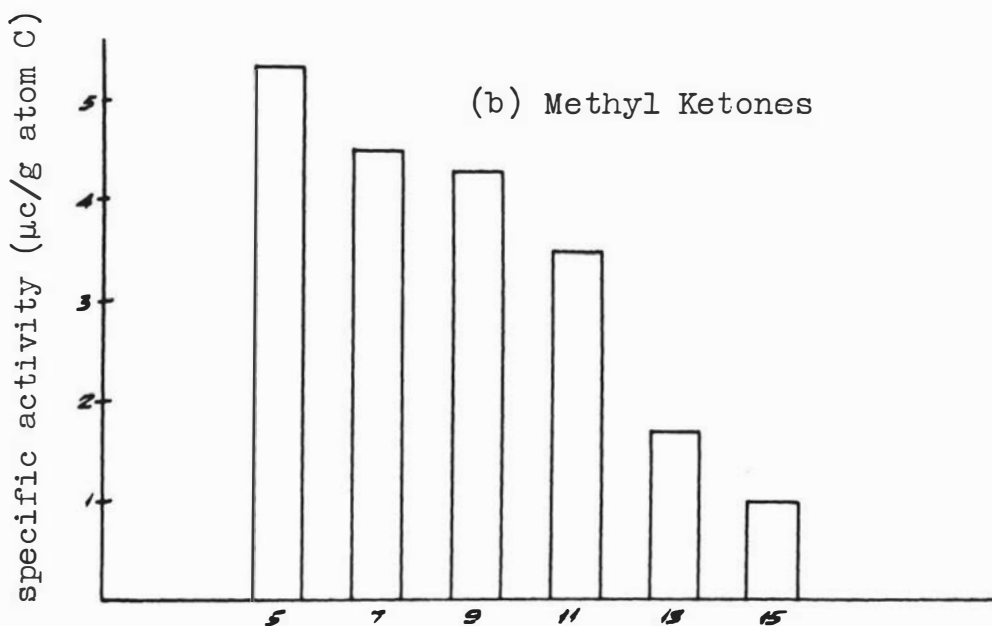
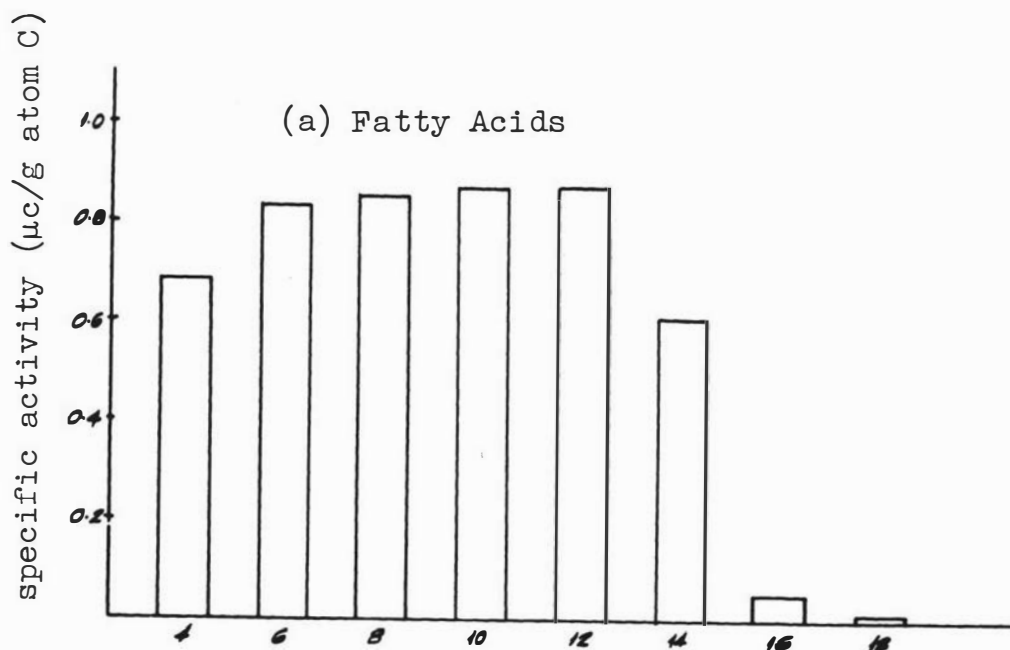


Fig.3. Relationship between specific activity and chain length of (a) glyceride fatty acids from milk fat of cow injected with 5 mc of carboxy - ^{14}C acetate 3 hours previously (b) methyl ketones found in steam distillates from same milk fat.

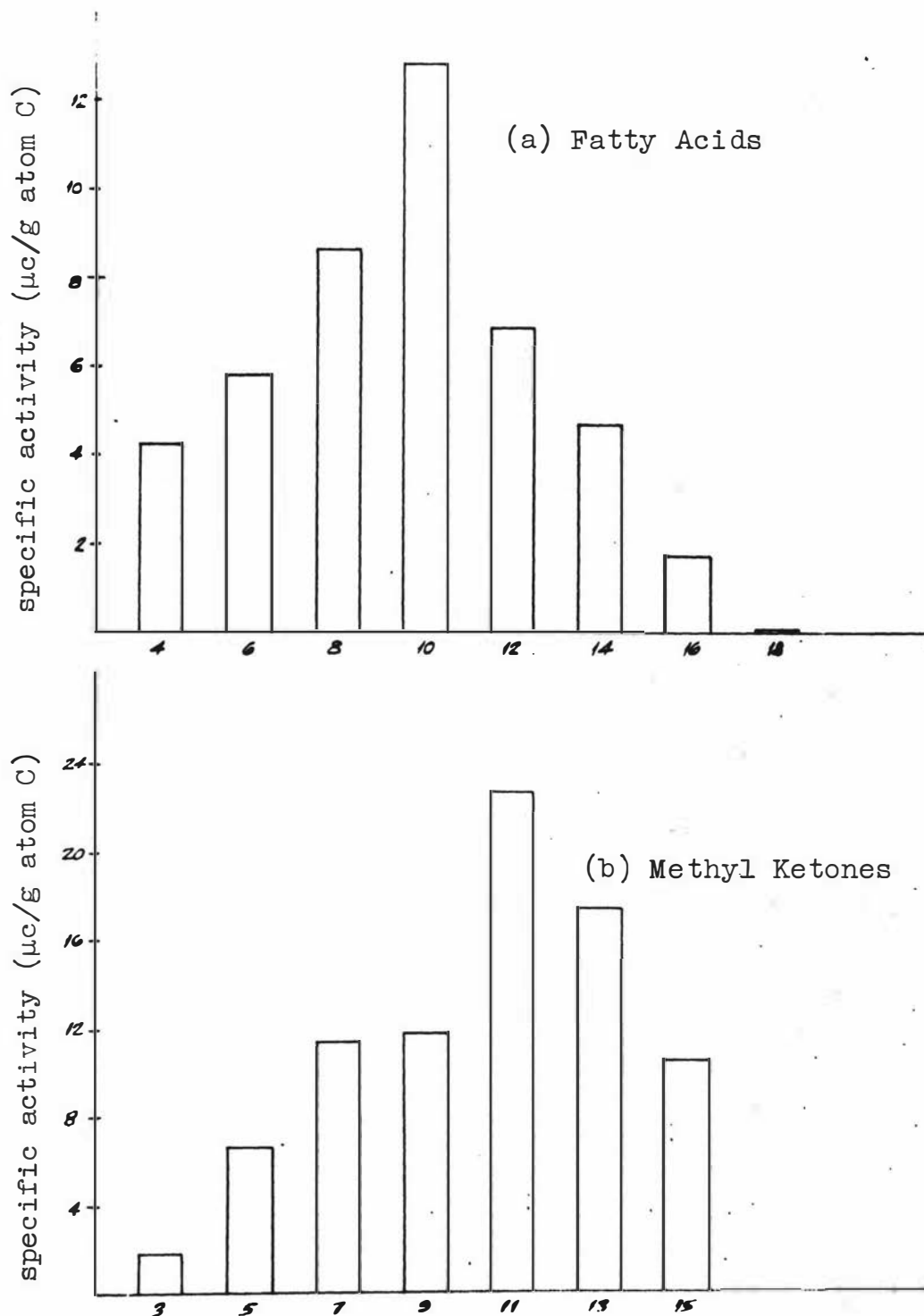


Fig.4. Relationship between specific activity and chain length of (a) glyceride fatty acids from milk fat of cow injected with 5 mc of carboxy - ^{14}C acetate 9 hours previously (b) methyl ketones found in steam distillates from same milk fat.

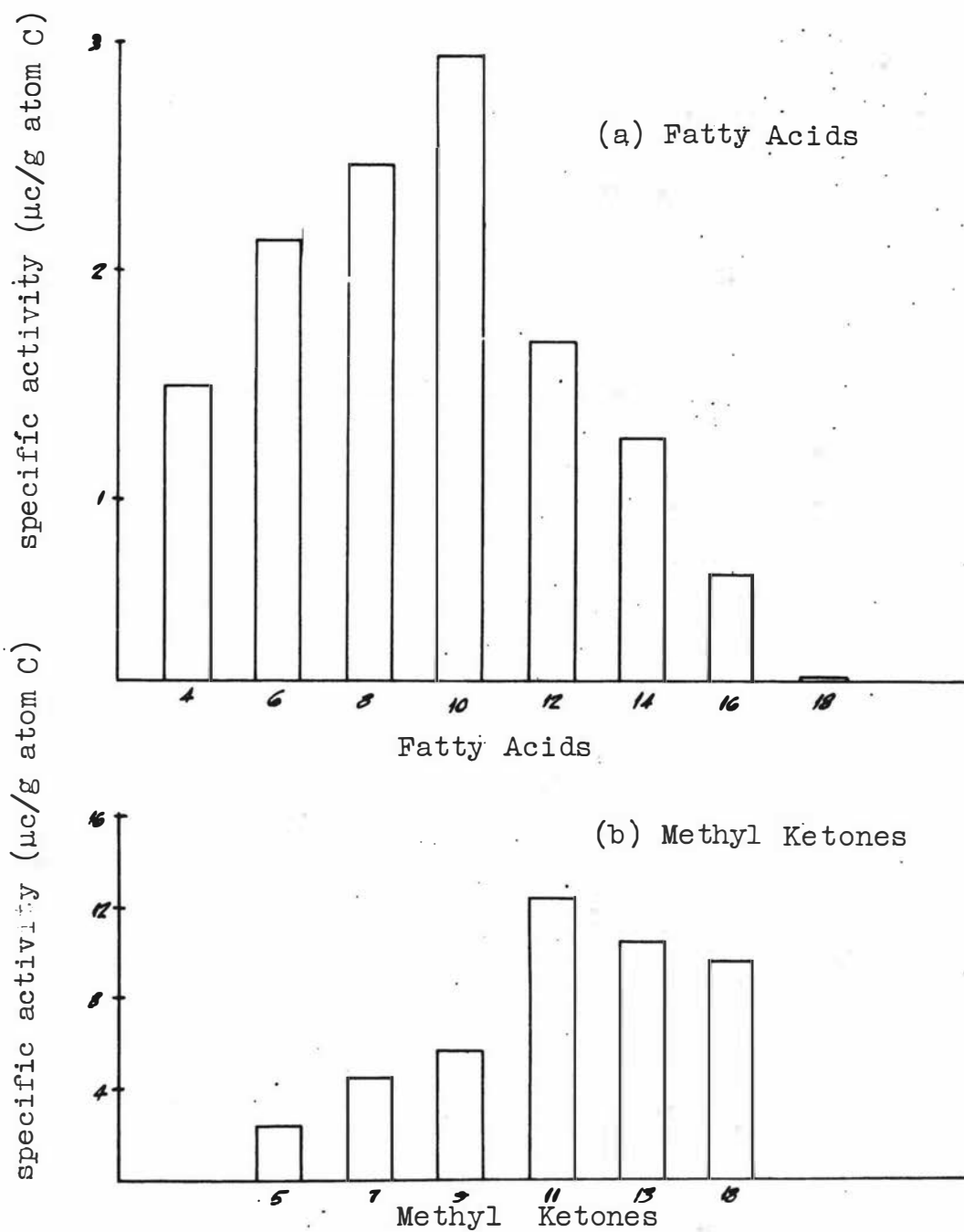


Fig. 5. Relationship between specific activity and chain length of (a) glyceride fatty acids from milk fat of cow injected with 5 mc of carboxy - ^{14}C acetate 22 hours previously (b) methyl ketones found in steam distillates from same milk fat.

Although appreciable proportions of acetone were obtained in the steam distillates of each milking, radioactivity was detected only in the acetone from the second milking and even in this sample the acetone had a very low activity. It was noteworthy that, in all 3 milkings, the only fatty acid with a specific activity greater than the corresponding methyl ketone was butyric acid.

The relative activities of the methyl ketones were confirmed qualitatively by preparing autoradiographs of the paper chromatograms used to separate and identify the 2,4-DNP hydrazones obtained from the 2nd and 3rd milkings.

¹⁴C labelling patterns of fatty acids

The fatty acid composition of the milk fat as shown by relative peak areas on gas chromatograms fell within the range normally found (e.g. Hawke, 1957). In addition propionic acid and a C₅ acid, assumed to be valeric acid, were also detected in trace amounts but their activities were not determined.

Figs. 3 - 5 show the distribution of ¹⁴C among the fatty acids of milk fat obtained from the three milkings. The fatty acids from the fat of the first milking (3 hours after injection) had low specific activities, the C₆ - C₁₂ acids showing maximum activity (about 0.85 μc/g atom C). The most radioactive fatty acids were recovered from the second milk sample 9 hours after injection, peak activity being

found at C₁₀ (12.9 µc/g atom C).

The pattern of labelling of the fatty acids from the third milking, 22 hours after injection, was almost identical to the 2nd milking, with peak activity again at C₁₀ acid (2.94 µc/g atom C). The specific activities were however 3 to 4 times lower than in the second milking whereas the decrease in specific activities of the methyl ketones from the 2nd to the 3rd milking was only 2 to 3 times.

In all 3 milkings the "odd" numbered fatty acids C₁₅ and C₁₇ n-saturated acids had specific activities very similar to those of the C₁₄ and C₁₆ acids (Table 7). On the other hand oleic and stearic acids had extremely low activities indicating that they were not synthesised to any significant extent from acetate. The activity of oleic acid from all 3 milkings was higher than that of stearic acid (Table 7), the reverse of expectation if the hypothesis that oleic acid is formed solely from stearic acid were true. On the other hand the C₁₆ unsaturated acid had a lower specific activity than the C₁₆ saturated acid in all 3 milkings in accord with recent theory of the formation of unsaturated acids in milk fat (Bloomfield & Bloch, 1960).

Table 7. The ^{14}C -labelling ($\mu\text{curies/g atom C}$) of fatty acids of milk fats from a cow milked 3, 9 and 22 hr. after intravenous injection of carboxy- ^{14}C acetate

Acid	Moles* %	1st Milking	2nd Milking	3rd Milking
C ₂	0.3	0.03	1.13	--
C ₄	9.1	0.69	4.18	1.49
C ₆	3.9	0.83	5.81	2.15
C ₈	1.8	0.85	8.56	2.44
C ₁₀	2.7	0.86	12.90	2.94
C ₁₂	2.9	0.87	6.89	1.69
C ₁₄	9.6	0.58	4.68	1.27
C ₁₅	1.1	0.18	1.10	--
C ₁₆	23.4	0.05	1.80	0.48
C _{16:1}	1.7	--	1.75	0.29
C ₁₇	0.8	--	0.97	--
C ₁₈	13.9	0.002	0.04	0.02
C _{18:1}	26.6	0.06	0.08	0.03

* Hawke (1957)

-- Not determined

Part II. METHYL KETONES FOUND IN STEAM DISTILLATES
FROM CHEESE CONTAINING SYNTHETIC TRIGLYCERIDES

The possibility existed that methyl ketones were being formed in Cheddar cheese from both a slow breakdown of bound β -keto acids in the milk fat and also from the β -oxidation of free fatty acids. An attempt was made to obtain evidence for the latter mechanism by incorporating into Cheddar cheese synthetic triglycerides containing fatty acids (heptanoic, nonanoic and undecanoic) which do not normally occur in milk fat in more than trace amounts. The assumption was made that the added synthetic triglycerides would be as susceptible to hydrolysis as the natural triglycerides in cheese fat since there is evidence for the non-specific nature of lipases in cheese (Stadhouders, 1956). If fatty acids were degraded to methyl ketones by β -oxidation, ketones with six, eight and ten carbon atoms respectively would be expected in the experimental cheeses in addition to the normal range of methyl ketones with an odd number of carbon atoms from C_3 to C_{15} .

The micro-organisms considered most likely to be responsible for the breakdown of milk fat to methyl ketones are fungi and yeasts. Preliminary experiments were carried out to ensure that Penicillium roqueforti (chosen as a general representative of lipolytic organisms that might be of importance in producing Cheddar flavour) was able to oxidise triglycerides containing fatty acids with an odd number of carbon atoms, at the pH's to be normally found in Cheddar cheese, as readily as those containing the even-numbered fatty acids.

Manufacture of cheeses containing synthetic triglycerides.

Triheptanoin, trinonanoin, and triundecanoin were prepared by the method of Malkin & Bevan (1957), emulsified separately in 1 litre skim milk and added to different vats of milk. From these "atypical" milks, three 40 lb cheeses were manufactured, each containing one of the above synthetic triglycerides. Two further 40 lb cheeses without added triglyceride were also made to serve as controls. The 5 cheeses were all manufactured from the same supply of milk.

The percentage amount of each "abnormal" fatty acid incorporated with the cheeses was determined by extracting samples of fat from each of the five cheeses when 14 days old. The methyl esters of the fatty acids were prepared by the method of Roper & Ma (1957) and analysed by vapour phase chromatography.

Isolation of carbonyl compounds

After 9 months ripening the five cheeses were analysed for methyl ketones. The experimental and the control cheeses were then judged to possess typical Cheddar flavour, although a superimposed "fatty" flavour was noted in the experimental cheeses.

The heptanoic, nonanoic and undecanoic acids incorporated into the 3 experimental cheeses as triglycerides were comparable in quantity (1.9, 1.2 and 1.3 moles % respectively) to the quantities of fatty acids of similar molecular weights normally present in cheese fat (e.g., octanoic 1.8 and decanoic 2.7 moles %). Thus if

methyl ketones are produced in Cheddar cheese by the enzymic oxidation of fatty acids, it was anticipated that the experimental cheeses would at maturity contain respectively C₆, C₈ or C₁₀ methyl ketones in quantities comparable to the "odd-numbered" ketones to be expected in both the experimental and the control cheeses. Analysis of the steam distillates at atmospheric pressure, however, showed no differences in methyl ketone pattern, the yields of 2-hexanone, 2-octanone and 2-decanone (approximately 0.01 mg per Kg cheese) obtained from cheeses containing trihexanoin, trioctanoin and tridecanoin respectively being no greater than from the control cheeses.

DISCUSSION OF

PARTS I and II

The results obtained in the present investigation show that methyl ketones are readily produced as artifacts during extraction procedures which involve the heating of dairy products containing milk fat. Thus on steam distillation at atmospheric pressure, cream, milk fat, cheese-oil and Cheddar cheeses from one day to 13 months old all gave the same range of methyl ketones with odd numbers of carbon atoms from C_3 to C_{15} . The persistence during distillation of these substances indicated that they were mainly being formed as artifacts.

Patton & Keeney (1958) identified 2-pentadecanone and possibly 2-tridecanone in the high melting glyceride fraction of milk fat, associated with the protein-phospholipid complex in the so-called membrane of milk fat globules. However, in general the rate at which the methyl ketones distil (Tables 3 and 4) would appear to suggest that they are not present in the free state since, as distillation proceeds, one would expect the exponential decrease in the concentrations of the methyl ketones that normally obtains for simple distillation procedures (Scott, 1955). This is approximately true only of 2-heptanone and not at all for the other methyl ketones.

While the present investigation was in progress, Bolding & Taylor (1962) also obtained a range of methyl ketones with an odd number of carbon atoms from C_7 to C_{15} by the steam distillation at 180° of milk fat from which volatile carbonyls had been removed completely by high vacuum degassing. Nawar, Cancel & Fagerson (1962) similarly detected the C_3 to C_{11} methyl ketones from milk fat that had been heated for 3 hours at 130° . As shown in Table 5 the concentrations of methyl ketones obtained by exhaustive steam

distillation of milk fat at 100° were in fair agreement with those given by Boldingh & Taylor (1962) for distillation of milk fat at 180°. These appeared to be the maximum concentrations of the methyl ketones obtainable by distillation and indicated that the precursors were present in relatively small amounts in milk fat. The average values given in Table 5 were also confirmed by totalling concentrations of methyl ketones found in the successive distillate fractions from cheese and milk fat (Tables 3 and 4). The close similarity of the values for cheese and milk fat suggested that the precursors of the methyl ketones in both products were identical.

The average yields of methyl ketones up to C₁₁ obtained by exhaustive steam distillation of different milk fat samples were C₇ (25 p.p.m.) > C₉ (16 p.p.m.) > C₁₁ (14 p.p.m.) > C₅ (13 p.p.m.). The radioactive milk fat samples in this investigation gave a lower yield of C₇ methyl ketone (14 p.p.m.) which was however still significantly higher than that of the C₉ (10 p.p.m.), C₁₁ (10 p.p.m.), and C₅ (9 p.p.m.) methyl ketones (Table 6). These results were similar to those of Parks, Keeney, Katz & Schwartz (1964) who obtained a yield of 2-heptanone (17 p.p.m.) which was roughly twice that of the C₅, C₉ or C₁₁ methyl ketones. Despite differences in total amount the relative abundance of the methyl ketones was much the same from all milk fats. Since the rates of formation of β-keto acids and their rates of conversion to fatty acids might be expected to determine the relative quantities of methyl ketones found, it is interesting to note that 2-heptanone was the most abundant of the C₅ to C₁₁ methyl ketones and the corresponding C₈ acid the least abundant of the C₆ to C₁₂ fatty acids. This inverse relationship may however be coincidental.

The detection in steam distillates of cheese and milk fat of traces of "even-numbered" methyl ketones from C_4 to C_{16} was in agreement with the isolation from milk fat of small quantities (about 2.1% of the total fatty acids) of the "odd-numbered" fatty acids from C_{11} to C_{17} (see Shorland, 1963). In this investigation the presence of C_3 and C_5 fatty acids was also noted. The two "even-numbered" methyl ketones present in greatest amount (C_6 and C_8) were presumably derived from intermediates in the synthesis of C_7 and C_9 fatty acids. Neither of these fatty acids has yet been isolated from milk fat.

The variation in yields of methyl ketones from different samples of milk fat was not unexpected since the fatty acid composition (particularly of the $C_6 - C_{14}$ acids) of different milk fats varies considerably, being dependent upon such factors as feed and plane of nutrition (Hansen & Shorland, 1952; Jack & Smith, 1956). If the methyl ketones and fatty acids have a common precursor such as β -keto acid it seems probable that the precursor and hence the yield of methyl ketones will also show a similar variation.

There appears to be no connection between the presence of β -keto acids and the large proportions of short chain ($C_4 - C_{14}$) fatty acids in milk fat. The steam distillation of coconut oil, which is a lipid containing a much higher proportion of $C_8 - C_{14}$ fatty acids (Hilditch, 1956), did not lead to the formation of any methyl ketones other than acetone. It must be assumed therefore that if any intermediates such as the β -keto acids are incorporated into the triglycerides of coconut oil, they are reduced in situ. The presence of

β -keto acids in milk fat might result therefore from their incorporation into the glyceride molecule and subsequent rapid removal from the site of synthesis.

The use of radioactive milk fat from a lactating cow which had been injected intravenously with carboxy- ^{14}C acetate allowed a direct comparison to be made between the labelling patterns of the fatty acids and the corresponding methyl ketones from the same milk source. The advantage of such a comparison was that two points on the biosynthetic pathway of milk fat (β -keto acid and the corresponding fatty acid) could be examined at the same time.

A very significant finding from all three milkings was the extremely low activity of C_{18} acid. This was in direct agreement with the fact that no C_{17} methyl ketone was detected in any of the numerous steam distillations of milk fat or cheese. Similarly little activity was found in C_{18} acid by Popják et al (1951) in milk fat from a goat after injection of labelled acetate, although Gerson, Hawke, Shorland & Melhuish (1960) found the specific activity of C_{18} acid to be almost as high as that of C_{16} acid in milk fat from a lactating cow which had been injected with carboxy- ^{14}C valerate. In the present investigation both C_{15} and C_{17} fatty acids had specific activities that were of the same order as those of the C_{14} and C_{16} acids (Table 7), in agreement with the detection of traces of the corresponding methyl ketones (C_{14} and C_{16}) in steam distillates from milk fat.

The generally accepted view (e.g. Folley & McNaught, 1961) is that, in the biosynthesis of milk fat (Dils & Popják, 1962) and other lipids (Wakil, 1961), the fatty acids up to and including palmitic acid are synthesised from an acetate pool whereas C_{18} acids and above are

synthesised by less direct pathways or from the blood triglycerides. The distribution of specific activities of fatty acids and methyl ketones from the radioactive milk fat used in this investigation was consistent with the origin of the milk fat from both sources. The similarity in labelling patterns together with the very low radioactivity found in the unsaturated C₁₈ fatty acids would appear to exclude the possibility, suggested by Wong et al (1958), that the methyl ketones are products of the autoxidation of milk fat.

Specific activities of both acids and methyl ketones from the fat of the first milking were low, confirming the observation of Rogers & Kleiber (1957) that a delay of several hours occurs between synthesis and secretion of milk fat. They suggest that this is more likely to be a delay in actual secretion of fat than either slow rate of actual synthesis or of slow transport of fat to the udder from other synthetic sites. Hardwick, Linzell & Mepham (1963) also observed the relatively slow rate of secretion of fat and fat precursors in the alveoli after its synthesis in the cells.

Specific activities of both fatty acids and methyl ketones from the second milking increased approximately to ten times the values in the first milking, the C₁₀ acid and C₁₁ methyl ketone respectively having the highest specific activities. This may be a reflection of the earlier formation of β -keto acids in the synthesis of fatty acids and suggests that they may be incorporated more rapidly than the corresponding fatty acids.

The fatty acids and methyl ketones from the fat of

the third milking had specific activities lower than those from the second milking, although the patterns of labelling were almost identical. The C₁₁ to C₁₅ methyl ketones contained the major part of the activity of the ketones and the C₄ to C₁₀ fatty acids were the most active acids in both the second and third milkings.

The decline in specific activities following the maximum at C₁₀ for the fatty acids and that at C₁₁ for the methyl ketones, and also the general decrease of the extent of labelling of the fatty acids and methyl ketones with time, can be explained by the replacement of labelled by unlabelled acetate and D-β-hydroxybutyrate during the course of synthesis (Popják, French & Folley, 1951). The dilution of ¹⁴C acetate with inactive acetate was supported in this investigation by the low specific activity of the acetate isolated from the milk fat.

The ¹⁴C labelling pattern of the fatty acids from the second milking was almost identical to that obtained by Popják et al (1951) in goat-milk fat during the 12 hr. period after injection of labelled acetate. The pattern from the third milking however differed from that found by Popják et al in goat-milk fat from milkings taken in the 12 - 48 hr. period after injection. The difference in species and the times of sampling presumably have contributed to these conflicting results.

While it is difficult to draw precise conclusions as to the origin of components of milk fat collected at a few points in time from the cow, the data obtained in this investigation would appear to support the conclusion of Patton & McCarthy (1963) that the supply of fatty acids in milk fat synthesis comes from at least two sources, which may and probably do intermingle to

a substantial degree; namely fatty acids transferred to the mammary gland from blood lipids (predominately $C_{12} - C_{18}$ acids) and the C_4 to C_{16} acids synthesised from acetate and D- β -hydroxybutyrate in the mammary tissue. Part at least of the blood lipids can be synthesised by the blood cells, whose ability to convert labelled acetate into all the common saturated and unsaturated long chain fatty acids (mainly C_{12} and above) was demonstrated by James, Lovelock & Webb (1959). They recovered labelled acids from both neutral fat and phospholipids of the plasma showing that, after synthesising these lipids, the cells secreted them into the plasma where they were incorporated into lipoproteins. McCarthy, Patton & Evans (1960) concluded that the blood triglycerides could provide all the oleate, linoleate, stearate, palmitoleate, myristoleate, 50% of the palmitate, 25% of the myristate and all the C_{10} fatty acid necessary for milk fat.

Gerson et al (1960) postulated two sites of fatty acid synthesis in the cow, the udder predominately synthesising the C_4 to C_{10} fatty acids while the C_{12} and above were synthesised at other sites from separate pools containing ^{14}C acetate. If this is true it is possible that the fatty acids from the various sites of synthesis enter the fatty acid pool at different rates. Fatty acids synthesised for instance in the blood or the liver may be transferred to body depot fats containing predominately long chain fatty acids (C_{12} upwards), which will turn over comparatively slowly into the blood stream and thence into the mammary gland. The $C_{12} - C_{16}$ fatty acids may possibly therefore be contributing to the fatty acid labelling pattern in milk fat in two ways : firstly by direct incorporation into

the triglyceride and secondly by degradation in the mammary gland to acetate and re-synthesis of short chain fatty acids. The degradation of long chain fatty acids in the mammary gland, an hypothesis postulated by Hilditch in 1937 but which fell into disrepute after the experiments of Popják et al (1951), might therefore be of particular importance since each degradative step would yield a 2-C fragment, which presumably would enrich the acetate pool from which fatty acid synthesis is occurring.

The similarity of the labelling patterns of the fatty acids and the corresponding methyl ketones with one less carbon atom, particularly in the 2nd and 3rd milkings, suggests that the C₆ to C₁₆ β-keto acids and the corresponding fatty acids have a common precursor (or that one is the precursor of the other) and are together incorporated into the triglycerides. There does not appear, however, to be a simple explanation for the finding that the specific activities of the fatty acids in all three milkings were significantly lower (2 to 5 times) than the activities of the corresponding methyl ketones. However, if the conversion from β-keto acids to fatty acids is rate limiting or, alternatively, if there is a greater rate of incorporation of the β-keto acids than the fatty acids into the triglycerides, the β-keto acids would be expected to have the higher specific activities. There is no ready explanation as to why the C₁₁ to C₁₅ methyl ketones in the second and third milkings have specific activities that

are relatively much higher than both the corresponding C_{12} to C_{16} fatty acids and the C_5 to C_9 methyl ketones.

Singh & Kumar (1963) have recently obtained evidence that the biosynthetic pathway of butyric acid in the lactating mammary gland of rabbits is independent of the acetyl-CoA carboxylase reaction, unlike the biosynthesis of the C_6 - C_{16} fatty acids. Popják et al, (1951) on the basis of earlier studies by Shaw & Knodt (1941) suggested that D- β -hydroxybutyrate might be the direct precursor of the butyrate moiety of milk fat and concluded that 40% of the C_4 acid was synthesised in the udder from acetate and 60% (presumably derived from D- β -hydroxybutyric acid) was absorbed from the blood stream. While Kumar et al (1959) have supported this view, others (e.g. see Garton, 1963) have doubted that the contribution of D- β -hydroxybutyrate to fatty acid synthesis proceeds via butyrate. The finding in the present investigation that only butyric acid of the C_4 to C_{16} fatty acids, in all three milkings, had a higher specific activity than the corresponding methyl ketone suggests that the acetone found in steam distillates of milk fat is formed from a compound derived almost entirely from a precursor other than acetate.

The quantitative abundance of acetone in all mixtures of methyl ketones obtained by steam distillation of milk fat and its accompanying low or undetectable radioactivity in the present investigation may have particular significance in any consideration of

rumen fermentation products as milk fat precursors. The low activity in the 2,4-DNP hydrazone of acetone can be explained by assuming that most of the acetone precursor, presumably acetoacetate, was derived from the oxidation of a non-isotopic source such as D- β -hydroxybutyrate. This would be in agreement with the finding of Black, Kleiber & Brown (1961) that the cow can metabolise butyrate via the classical β -oxidation pathway and the TCA cycle.

The presence of unlabelled acetoacetate but labelled higher β -keto acids in milk triglycerides following injection of labelled acetate could be explained by the pathway, based upon the mechanism proposed by Bressler & Wakil (1962), outlined in Scheme 1. Small amounts of β -keto acids in triglycerides with similar specific activity to fatty acids of the same carbon number implies that some dissociation of β -ketoacyl-enzyme occurs in the mammary gland which is similar to the dissociation of acyl-enzyme prior to incorporation into triglycerides. The quantitative importance of acetone in the mixed methyl ketones from milk fat suggests that more acetoacetate than any of the higher β -ketoacids is present in the metabolic pool.

The hypothesis that bound β -keto acids in the triglyceride molecules of milk fat are the precursors of methyl ketones would appear to have been established unequivocally by two other groups of workers. Van der Ven, Haverkamp Begemann & Schogt (1963) found that milk fat

yielded a series of six pyrazalones, corresponding to six even-numbered β -keto acids (C_6 up to and including C_{16}), when treated with Girard-T reagent. They estimated that non-volatile β -keto acid esters were present in a concentration of approximately 0.03% in milk fat. Parks et al (1964) confirmed this work by direct isolation from milk fat of the methyl ketone precursors which they demonstrated were triglycerides containing one β -keto acid and two fatty acid moieties. They concluded that these glycerides accounted for 0.045% of the milk fat.

The possibility that methyl ketones were being formed in Cheddar cheese from the β -oxidation of free fatty acids as well as from a slow breakdown of bound β -keto acids in the milk fat was shown to be improbable. Synthetic triglycerides of fatty acids (undecanoic, nonanoic, and heptanoic), which occur normally only in traces in milk fat, were incorporated in Cheddar cheeses. On steam distillation of these cheeses when mature, no significant amounts of methyl ketones with one less carbon atom than the fatty acids of the synthetic triglycerides were detected although the "odd-numbered" $C_3 - C_{15}$ methyl ketones to be found in distillates of normal cheese were obtained. Since also P. roqueforti was unable to oxidise C_{14} and C_{16} fatty acids to the corresponding methyl ketones, the C_{13} and C_{15} methyl ketones detected probably originated from the C_{14} and C_{16} β -keto acids respectively of milk fat. This agreed with the earlier finding that the maximum yield of methyl ketones obtained by exhaustive steam distillation of mature Cheddar cheese was not greater than that obtainable from a comparable weight of fresh milk fat. It is perhaps also significant that

Bassett & Harper (1958) did not isolate free β -keto acids from Cheddar cheese, although such intermediates in fatty acid oxidation were found in Blue cheese. The evidence generally suggests, therefore, that methyl ketones found in Cheddar cheese are not formed by oxidation of fatty acids to β -keto acids and subsequent decarboxylation, but only by the breakdown of β -keto acids already present in the triglyceride molecule.

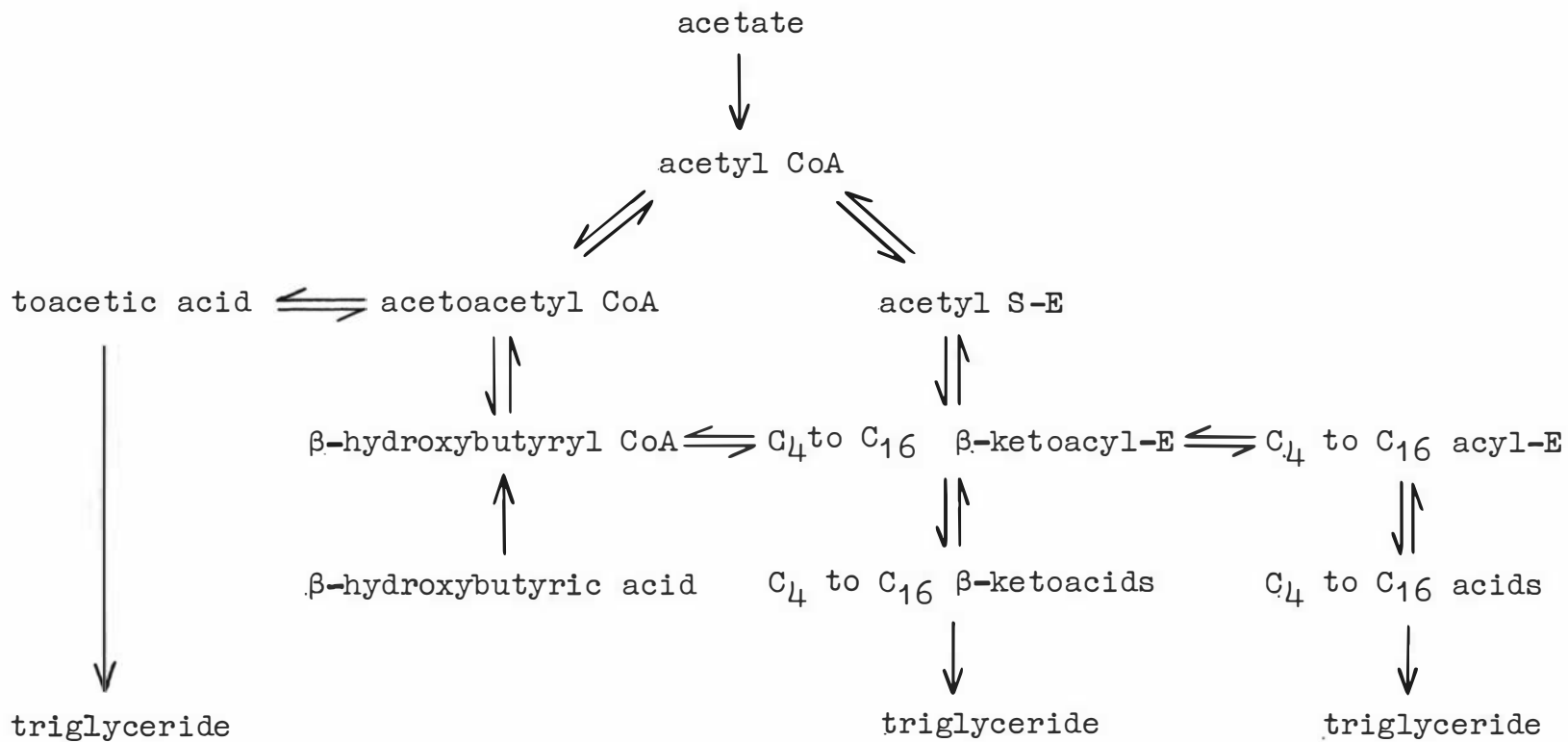
Although the chemical nature of Cheddar cheese flavour is still unknown there is evidence to suggest that methyl ketones are partly responsible (e.g. Walker, 1961) either in free form or possibly combined with other flavourful compounds such as hydrogen sulphide. It is also not known whether the natural enzymes in milk, the rennet enzymes or the bacterial flora are responsible for the development of Cheddar flavour. If methyl ketones are responsible, at least in part, for the development of Cheddar flavour, one can explain the retarding of the flavour development through pasteurisation as the partial destruction of natural milk lipases or microbial lipases capable of hydrolysing the β -keto acid moiety of milk fat. The addition of cultures of lipolytic bacteria such as micrococci to pasteurised cheese milk considerably enhanced the flavour of the Cheddar cheese made from it (Robertson & Perry, 1962), which would be expected by the above theory. The addition to cheese milk of less strongly lipolytic bacteria such as lactobacilli has a lesser effect, although a positive one, on Cheddar flavour development. Similarly the limited amount of β -keto acid precursor available might explain the observation of Robertson & Perry (1962) that there appeared to be

a limiting concentration of flavour forming substrate since increasing the size of the inoculum of micrococcus culture from 2% to 10% did not accelerate the rate of or extent of flavour formation.

It has been suggested (Harvey & Walker, 1960) that the time of first appearance of certain methyl ketones above C₅ coincided with the appearance of the typical Cheddar flavour. Since such ketones were found not only in distillates from cheese of any age but also from fresh milk fat it is evident that this is not the case. The present investigation has shown that it is unlikely that a homologous series of methyl ketones is formed by microbiological action during Cheddar cheese ripening. Since, however, methyl ketones are obtained from mature cheese at room temperature, the breakdown of precursors which occurs rapidly on heating of milk fat may also take place slowly at ripening temperature, either by enzyme action or by a purely physico-chemical process. This would result in the formation of relatively small quantities of a range of methyl ketones as cheese matures.

It is evident, however, that new methods will have to be employed in the quantitative examination of cheese in order to differentiate between methyl ketones present as such in ripening cheese and those formed as artifacts during the extraction. The further question of whether they play any part in cheese flavour then has still to be answered.

eme 1. A hypothetical mechanism for the formation and incorporation of β -keto acids into milk fat



Part III THE OXIDATION OF FATTY ACIDS AND
TRIGLYCERIDES BY PENICILLIUM ROQUEFORTI

There are two aspects to the study of the physiological and biochemical properties of fungal mycelium (1) that connected with the effect of cultivation and growth and (2) that concerned with the biochemical activities of the cell material under conditions where growth, in the common sense of the term, does not take place. The latter utilises pre-formed cell material and is merely a logical extension of growth experiments. Of previous investigations into the uptake of oxygen by *Penicillia* in the presence of fatty acids, only two (Rolinson, 1954; Girolami & Knight, 1955) have made use of pre-formed cell material. The interpretation of the results of other workers has been made difficult by the complicating effects of growth.

It is well known that fungi are particularly sensitive to imposed environmental conditions which can lead to very marked alteration in metabolism. Much evidence has accumulated since the original study of Kluyver & Perquin (1933) to show that the biochemical behaviour of fungal cell material directly depends on the environmental conditions of the organism during its growth phase. It was shown for example that glucose oxidase activity in *Aspergillus niger* was considerably higher in media with low phosphate concentration (Mann 1944). Horiuchi, Horiuchi & Mizuno (1959) and Torriani (1960) have also demonstrated that certain phosphatases are formed only in measurable amount when inorganic phosphate becomes limiting. Darby & Goddard (1950) have pointed out that the wide differences existing between the results dealing with fungal respiration from various workers are possibly more to be ascribed to environmental differences rather than to real differences in physiology of the organisms concerned.

Meyers & Knight (1958) developed a synthetic medium for growth of P. roqueforti but growth was extremely poor unless the initial pH was adjusted to 4. The medium contained both acetate and oleate and was thus not suitable for the growth of mycelium to be used subsequently in fatty acid studies. It was therefore necessary to develop a new synthetic medium in which fatty acids were not required for growth.

As Foster (1949) has pointed out, a characteristic of fungal metabolism is the inconstancy of the metabolic activities of fungi. A true biochemical evaluation of a particular fungus can be secured only by study of its metabolism under different conditions, the extremes and one intermediate of any one factor usually being sufficient to picture the fungus as a metabolic entity. In this investigation therefore the oxidation of the homologous series of fatty acids over a range of pH by both mycelium and spores of P. roqueforti was studied.

CULTURES

Two strains of Penicillium roqueforti were used throughout these studies. The first (6989) was obtained from the American Type Culture Collection, Washington. A second strain, isolated from N.Z. Blue cheese, was identified by Dr. Barbara Latch of Massey University as Penicillium roqueforti Thom.

The fungus was usually sporulated on slopes of Czapek-Dox agar for 4 - 14 days, although in preliminary experiments potato dextrose agar and malt agar slopes were used. To harvest the spores, distilled water or buffer was added to each tube and the spores suspended in the liquid as they were carefully scraped from the surface of the slope with a sterile needle. The spore suspension was used immediately.

Standardisation of spore suspension

A spore suspension was standardised in a haemocytometer to a concentration of 1×10^7 spores per ml. The turbidity of each spore suspension subsequently used was measured against this standard in a Beckman DU spectrophotometer at 325 m μ and diluted to the concentration of the standard.

MATERIALS

Acids. Fatty acids with an even number of carbon atoms (99.8 +% pure) were obtained from Applied Science Laboratories, Inc., State College, Pa; acids with an odd number of carbon atoms from L. Light & Co. Ltd., England; the synthetic triglycerides from Eastman Organic Chemicals, Rochester, N.Y. The salts of the fatty acids were made by the addition of potassium hydroxide to pH 7.3 and making up to the required concentration with distilled water. Salts of acids with more than 12 carbon atoms were warmed to 45° before addition to the side arm of a Warburg flask to ensure that all the material was in solution.

METHODS

It was found that reproducible results were obtained only by standardising the method of growing the mycelium and using it under controlled and defined conditions.

Growth of mycelium

The spores were grown on slopes of Czapek-Dox agar (Oxoid preparation) at 22°C. After 5-6 days growth the spores were transferred to 100 ml of the growth medium in

500 ml Erlenmeyer flasks. The spores were allowed to germinate, without shaking, overnight followed by shaking for 48 hours at 20° on a microid flask shaker (Griffen & George Ltd.). This prevented the clumping together of large individual colonies and resulted in a uniform and homogeneously suspended type of culture. In such a situation all cells have comparable nutritional conditions and equal access to oxygen. It was possible to duplicate experiments and to obtain reasonable agreement by following the above procedures which gave results comparable with the re-inoculation method of Foster (1949) with considerably less chance of contamination. The cells were harvested by filtration on a Buchner funnel, resuspended several times in about 2 litres of distilled water and refiltered. The mycelium was finally suspended in phosphate buffer and, in most experiments with whole cells, blended gently in a "Polytron" homogenizer (Mobil Aaran A.G, Aahran, Switzerland).

Gently blended mycelium resulted in a slightly greater oxygen uptake than whole cells, in contrast to the decrease reported by Darby & Goddard (1950) working with Myrothecium Verrucaria, and was used in all experiments reported unless otherwise stated.

After shaking for 48 hours the pH fell to about 2.5, being accompanied on occasions by spore formation. For this reason the growth period was seldom continued after 48 hours. Spore formation in submerged culture is very unusual (Cochrane, 1958) but both strains of P. roqueforti demonstrated this phenomenon.

The concentration of blended cells was adjusted to approximately 10 mg (dry weight) per ml, the exact concentration being determined by dry weight

measurement. The rates of oxygen uptake were usually calculated on 10 mg dry weight. Oxygen uptake measurements were made manometrically at 27°C in a standard Warburg apparatus (Braun, Model "V8"). 0.2 ml 20% KOH was used in the inner cell of the Warburg flasks unless otherwise stated.

Oxygen uptake by spores

The measurements were made with spores suspended in water or buffer after removal from their parent cultures. The spores were never observed to germinate in water or buffer although very slight mycelial growth was usually noted after about 6 hours when incubating spores with fatty acids in buffer. Care had therefore to be taken when interpreting oxygen uptakes, particularly as the endogenous respiratory activity of the spores was fairly close to the lower limits of sensitivity of the standard Warburg techniques.

At the completion of the oxygen uptake experiment 1 ml of the contents of the Warburg flask was pipetted into 1 ml of 2,4-DNP hydrazine (2 g in 1 litre of 2N HCl) in a stoppered test tube. Methyl ketones were identified and their concentrations determined as described below.

THE COMPOSITION OF THE SYNTHETIC GROWTH MEDIUM AND
SUBSEQUENT OXYGEN UPTAKE OF THE MYCELIUM IN THE PRESENCE
OF FATTY ACIDS

A synthetic growth medium was developed that would lead to extensive growth of a mycelium which was capable of good oxygen uptake and methyl ketone formation when incubated with fatty acids as sole carbon substrates. These initial experiments were carried out with octanoic acid.

Effect of phosphate concentration. Reported optimum phosphate concentrations for other fungi have been of the order of 0.001 M. It was found however that the growing mycelium rapidly utilised the phosphate with consequent loss of buffering capacity, the pH falling below pH 3.0. The phosphate concentration was increased to 0.03 M but even this concentration failed to hold the pH steady for more than 48 hours.

Effect of magnesium. Varying the concentration of magnesium in the growth medium had little effect on mycelial growth or its subsequent biochemical performance. Spores concentrate magnesium from the medium on which they are grown (Foster, 1949) and presumably contain sufficient for mycelial growth.

Effect of calcium. The presence of calcium in the growth medium did not lead to a significant increase in oxidative ability of the mycelium. Since also calcium is not considered to be an essential element for mycelial growth (Cochrane, 1959), it was not

included in the final growth medium.

Nature of nitrogen source. In early experiments ammonium sulphate was used but it was found subsequently that ammonium nitrate gave better growth of mycelium and higher oxidising ability. Some fungi, e.g. certain strains of Aspergillus niger, have been shown to selectively utilise NO_3 at pH's below 4.0 (Cochrane, 1958).

Effect of initial pH of growth medium

Oxygen uptake by mycelium in the presence of fatty acids at pH 6.3 was a maximum when the mycelium had been grown in a medium which had an initial pH of about 6.3 (Table 8).

Growth medium finally adopted. After further preliminary trials the following synthetic medium was used to grow mycelium from spores: 2 g ammonium nitrate; 0.15 g magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$); 0.25 g potassium chloride; 0.0125 g ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). These 4 components were dissolved in 400 ml water and autoclaved for 20 min. at 12 p.s.i. The resultant brown precipitate was allowed to settle, the supernatant being decanted after 24 hours. A solution of 10 g glucose, 0.6 g potassium dihydrogen phosphate and 0.4 g di-potassium hydrogen phosphate in 100 ml water (Seitz filtered in preference to autoclaving to safeguard against heat decomposition) was added. 1 ml of a trace element supplement solution (containing per litre :

Table 8. The effect of the initial pH of the growth medium on subsequent ability of mycelium to oxidise octanoic acid (1.5 μ moles)

Initial pH of growth medium	Oxygen Uptake (μ l)	
	Endogenous	Octanoic acid
4.1	80	141
4.5	83	185
5.3	120	330
5.8	135	381
6.3	102	402
6.7	110	370

(Each Warburg flask contained approximately 10 mg blended mycelium, 48 hours old, 30 μ moles phosphate buffer pH 6.3, distilled water to 3 ml. Period of incubation 120 minutes).

180 mg zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$); 15 mg copper nitrate, $\text{Cu}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$; 80 mg manganese nitrate, $\text{Mn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, and 10 mg ammonium molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, was also added. The pH of the final growth medium was 6.5.

Effect of carbon and nitrogen sources in growth medium on ability of mycelium to oxidise fatty acids

The synthetic medium described was compared with a variant in which the glucose and ammonium nitrate were replaced by casamino acids. In an attempt to determine whether specific β -keto acid decarboxylases were inducible, C_{12} acid was also added to two batches of growth media. The four media compared were thus:

Medium A.	Synthetic medium as described above		
" A + C_{12}	" "	+ C_{12} acid (2 $\mu\text{moles/ml}$)	
" B	" "	but glucose and ammonium nitrate replaced by casamino acids (3g/500ml)	
" B + C_{12}	As medium B + C_{12} acid (2 $\mu\text{moles/ml}$)		

Growth of mycelium was stronger in the media to which C_{12} acid had been added. As the results in Table 9 show however the addition of C_{12} acid to the growth medium did not induce a specific β -keto lauryl acid decarboxylase. On the contrary such mycelium showed less ability to oxidise C_{12} acid to 2-undecanone, and to a lesser extent C_8 acid to 2-heptanone, than mycelium which had been grown in absence of C_{12} acid. The fact

Table 9. The effect of composition of growth medium on the ability of mycelium to oxidise fatty acids

Medium*	Oxygen Uptake (μ l)			μ moles 2-heptanone from C ₈ acid	μ moles 2-undecanone from C ₁₂ acid
	Endogenous	3 μ moles C ₈ acid	3 μ moles C ₁₂ acid		
A	293	496	253	1.8	0.40
A + C ₁₂ acid	373	653	176	1.0	0.10
B	414	609	51	1.1	0.15
B + C ₁₂ acid	270	350	20	0.92	0.08

(Each Warburg contained approximately 10 mg mycelium, 50 hours old, 50 μ moles phosphate buffer pH 5.2. Period of incubation 135 minutes.)

* Medium A contained glucose and ammonium nitrate
 " B " casamino acids.)

that methyl ketones were formed in two very different growth media would indicate that the β -keto acid decarboxylases are constitutive enzymes. The presence of C_{12} acid in the growth media did not prevent the mycelium from oxidising C_8 acid readily although it was unable to oxidise C_{12} acid.

Mycelium grown in casamino acid media was much more sensitive to subsequent addition of C_{12} acid than that grown in a glucose/ammonium nitrate medium. The highest yield of ketone was obtained in the latter medium. Since the main aim of the investigation was to examine methyl ketone formation this medium was used in all subsequent work unless otherwise stated.

Estimation of micro quantities of methyl ketones as 2,4-DNP hydrazones

In Part I mention was made of the difficulties involved in the estimation of methyl ketones as 2,4-DNP hydrazones because of the apparent non-quantitative nature of the reaction with 2,4-DNP hydrazine reagent. In estimating methyl ketones as products of the oxidation of fatty acids by P. roqueforti, the quantities involved were much smaller, of the order 0.1 μ moles upwards. A colorimetric assay for small quantities of 2-heptanone has been reported (Haidle & Knight, 1960), in which the ketone was steam distilled and reacted with 2,4-DNP hydrazine reagent, NaOH added and the coloured complex that developed measured spectrophotometrically. Knight (1963) however stated that the method was not entirely satisfactory.

In view of the large number of methyl ketone estimations to be carried out it was necessary to determine the reasons for the low recoveries of methyl ketones as their 2,4-DNP hydrazones and then to devise a method that was not only more quantitative but much less time consuming than the above steam distillation method.

Instability of 2,4-DNP hydrazones

The 2,4-DNP hydrazones of methyl ketones were found to decompose slowly in air after their formation, the equilibrium

$$\text{methyl ketone} + 2,4\text{-DNP hydrazine} \rightleftharpoons 2,4\text{-DNP hydrazone}$$

presumably moving to the left with time on account of evaporation of the volatile ketones. The stabilities of the 2,4-DNP hydrazones were determined by adding 1 ml aliquots of standard ketone solutions (0.75 μ moles per ml) to 1 ml 2,4-DNP hydrazine reagent in a number of 50 ml beakers. At the same time the reaction was carried out in stoppered test tubes. At intervals the 2,4-DNP hydrazones were extracted with carbonyl-free hexane as detailed later. In all cases the yield of 2,4-DNP hydrazones determined in the test tubes was virtually quantitative and of the order of 30% higher than that calculated from the first beaker which had been standing in the air for 30 minutes (Table 10). The yield of 2,4-DNP hydrazone from the beakers further slowly decreased with time, those of the C₃ to C₇ methyl ketones being the least stable.

Table 10. Recoveries of methyl ketones (0.75 μ moles) as the 2,4-DNP hydrazones from stoppered test tubes and from open beakers

	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	C ₉	C ₁₀	C ₁₁
<u>Stoppered</u>									
<u>Test tubes</u>									
30 min.	0.71	0.73	0.72	0.75	0.74	0.71	0.72	0.75	0.65
20 hrs.	0.69	0.71	0.72	0.75	0.70	0.71	0.72	0.75	0.68
<u>Beakers</u>									
30 min.	0.38	0.45	0.54	0.59	0.58	0.51	0.61	0.68	0.58
3 hrs.	0.17	0.24	0.48	0.51	0.48	0.47	0.54	0.64	0.56
5 hrs.	0.09	0.12	0.45	0.47	0.42	0.43	0.50	0.63	0.56
20 hrs.	Nil	Nil	0.23	0.25	0.23	0.28	0.34	0.53	0.48
% decrease in 19½ hrs.	100	100	58	58	60	45	44	22	18

(1 ml standard ketone solution added to 1 ml 2,4-DNP hydrazine reagent. Details of extraction given in text.)

Since it was apparent that the low recoveries of methyl ketones obtained in Part I were a result of their volatility the following method of estimation was developed which cut down the number of operations (detailed in Part I) leading to the final determination of the 2,4-DNP hydrazones in a spectrophotometer.

Method of estimation finally adopted

It was found that if 1 ml of the standard ketone solution were reacted with 1 ml of 2,4-DNP hydrazine in a stoppered test tube and allowed to stand for 30 minutes, 10 ml of carbonyl-free hexane extracted the ketone virtually quantitatively as its 2,4-DNP hydrazone. After vigorous shaking in the test tube the aqueous and hexane layers were allowed to settle, the upper hexane layer poured directly into the Beckman cell and its absorption read at 345 m μ . A "blank" was obtained by shaking 1 ml of 2,4-DNP hydrazine reagent with 1 ml of water and 10 ml carbonyl free hexane. After subtracting the "blank" from the reading of the test solution, the concentration of the methyl ketones could be obtained directly from standard curves prepared relating absorption at 345 m μ to the concentration of 2,4-DNP hydrazone.

Before the estimation was applied to the oxidation of fatty acids and triglycerides by spores and mycelium it was demonstrated that no carbonyls other than methyl ketones were formed in detectable amounts.

A natural corollary to the above observations on the volatility of methyl ketones in aqueous solution

was the need to carry out all incubations in stoppered vessels. The observation by Thaler (1949) that the formation of methyl ketones was "zig-zag" was confirmed both in plugged flasks and with stoppered bottles. However the recovery of methyl ketones from stoppered bottles was invariably greater and it is possible that part of the variation and low yields of other investigators resulted from evaporation of methyl ketone as it was formed.

Quantitative estimation of methyl carbinols in the presence of methyl ketones

At appropriate time intervals 1 ml of the medium was analysed for methyl ketone by the method described above using 2,4-DNP hydrazine reagent. At the same time 10 ml of medium were withdrawn, made alkaline until red to phenolphthalein, and extracted with diethyl ether. The extract was concentrated to approximately 0.5 ml by carefully removing the ether in a stream of air. 1 μ l of the concentrate was analysed in a gas chromatograph (Aerograph Hy-Fi, Model A-600-B, Wilkins Instrument & Research, Inc.). Columns normally used were either 20% UCON 50 HB 2000 or 20% Carbowax 1540 on Chromosorb W (Mesh 30-60), although initially 20% diethylene-glycolsuccinate (D.E.G.S.) on Firebrick was used.

Identification of the peaks was accomplished by co-chromatography with authentic samples of 2-heptanol and 2-heptanone. The retention times of 2-heptanol compared to that of 2-heptanone were 1.45 on the DEGS column (115°), 1.78 with Carbowax (90°) and 2.02 with

UCON (100°) columns. Quantitative measurements were made by measuring the areas under the curves on the chromatograms with a planimeter. Since the concentration of 2-heptanone was known accurately that of 2-heptanol could also be determined. Recovery trials of known amounts of 2-heptanone and 2-heptanol carried out using the procedure above were quantitative ($\pm 5\%$) over a range of concentrations of the alcohol and ketone from 0.25 - 10 μ moles per ml.

Further characterization of any 2-heptanol formed in these investigations was accomplished by forming the 3,5 dinitrobenzoate of the alcohol following the procedure of Holley & Holley (1952). The alcohol was identified by co-chromatography with the authentic 3,5 dinitrobenzoate of 2-heptanol using a modification of the paper chromatographic method of Sundt & Winter (1957). The 3,5 dinitrobenzoates were easily detectable as violet spots after 10 minutes irradiation under ultra violet light. The yield of 3,5 dinitrobenzoate from the secondary alcohol was however low and could not be used as a quantitative method.

Identification of free fatty acids

A modification of the method of Harper (1953) was used to determine the formation of free fatty acids in the incubation medium during the oxidation of triglycerides by spores.

A silica gel column was prepared in two sections using a chromatographic column, 35 mm I.D. by 250 mm in length with a perforated glass disc.

Bottom section. 5 g dry silicic acid (Mallinckrodt No.2487) were mixed thoroughly with 3 ml of 2M K_2HPO_4 - KH_2PO_4 buffer (pH 6.5) and slurried with 20 ml washed chloroform.

Top section. The sample, normally 10 ml, was acidified to pH 1.8 - 2.0 (pre-determined with 20% (v/v) H_2SO_4), 5 g silicic acid being added for every 3 ml medium. The mixture was ground thoroughly, slurried with 5% butanol in chloroform and transferred quantitatively on top of the bottom section.

Estimation of acids. The acids in the eluate were titrated with 0.02N NaOH after the addition of 10 ml neutral absolute alcohol and 0.1 ml of phenol red indicator. Standard colours were used to facilitate detection of the end point.

The reliability of the method was established by determining the recovery of known acids (butyric, hexanoic and octanoic) added individually to the medium prior to acidification. Two trials were made in which the concentration of added acids was 10 and 60 microequivalents. All the acids were quantitatively recovered by elution with 100 ml of the 5% butanol in chloroform solvent.

Blanks. The free fatty acid content of the commercial samples of triglycerides used was determined by running aliquots of the incubation media, before the addition of spores, down the column. Trihexanoin contained no free fatty acid, tributyrin and trioctanoin sufficient to account for only a very small percentage

of the ketones formed from them.

Measurement of $^{14}\text{CO}_2$ during respiration in Warburg flasks

The advantages of using liquid scintillation techniques over other methods for the measurement of radioactive carbon dioxide have been discussed by Rapkin (1962).

The hydroxide of Hyamine 10-X, (p-di-isobutyl-cresoxyethoxy-ethyl)-dimethyl benzyl ammonium hydroxide, was the first (Passman, Radin & Cooper, 1956) and is still the most widely used trapping agent for $^{14}\text{CO}_2$. More recently Wakil (1962) has used Hyamine impregnated filter paper directly in his Warburg vessels, which traps the liberated carbon dioxide. The paper is then removed, dried, transferred to a counting vial and counted with scintillation solvent without any additional manipulation. Similarly Chiriboga and Ray (1962) have described the same technique using KOH rather than Hyamine.

Efficiency of trapping agents.

Preliminary experiments with Whatman No.50 paper impregnated with 0.05 ml 20% KOH showed reproducibility of $^{14}\text{CO}_2$ recoveries to be fair only. It was considered that the use of DEAE-cellulose paper (Whatman DE 20, free base form), impregnated with 20% KOH, might more

firmly absorb the $^{14}\text{CO}_2$, the method being adapted from that of Sherman (1963) for enzyme assays. A comparison of the recovery of $^{14}\text{CO}_2$, liberated from a standard $\text{NaH}^{14}\text{CO}_3$ solution by 10% H_2SO_4 , showed that the Whatman DE 20 paper gave consistently higher and more reproducible results than the Whatman 50 paper (Table 11).

Table 11. The recovery of $^{14}\text{CO}_2$ by the use of Whatman No.50 and DE 20 papers impregnated with 0.05 ml 20% KOH

Trial	Theoretical Counts	$^{14}\text{CO}_2$ (c.p.m.)	
		What.50	What.DE20
1	22,800	21,860	21,600
	45,600	41,320	40,650
	68,400	56,420	63,300
2	4,560	3,185	4,045
	9,120	6,095	8,040
	13,680	8,165	12,310
	"	10,620	12,460
	"	10,060	12,440

Synthesis of non-available substrates

2-C¹⁴ octanoic acid, 2-heptanone-1-C¹⁴ and 2-heptanol were not available from commercial sources and had to be synthesised.

Synthesis of 2-C¹⁴ octanoic acid. The method used was a micro adaptation of that of Reid & Ruhoff (1944) for the synthesis of nonanoic acid. 0.3 g clean sodium was slowly dissolved in 7 ml butanol which had been dried over solid K₂CO₃. 0.2 mc of 2-C¹⁴ diethyl malonate, diluted to 2 ml with cold diethyl malonate, were added and the solution heated to 80-90°. 2 ml of 1-bromohexane were added slowly, refluxed for 2 hours, followed by 2 g KOH in 2 ml H₂O and the solution refluxed for a further 5 hours. The contents of the flask were then steam distilled and 20 ml of distillate containing the butanol were collected. 3.5 ml of conc. hydrochloric acid were added to the residue and the mixture refluxed for 1 hour. After cooling, the aqueous layer was removed with a pipette. The residue was now refluxed in an oil bath at 180° for 2 hours until CO₂ evolution had ceased. The octanoic acid was decanted from the solid residue and dried over anhydrous Na₂SO₄, the yield being approximately 15% (0.3 g).

The purity of the acid was checked directly by co-chromatography with a pure sample of octanoic acid on a gas chromatograph using a column packing of 20% (w/w) diethylene glycol succinate and 2% H₃PO₄ (85% H₃PO₄) on 60-80 mesh, acid washed "Celite 545". (Metcalf, 1960). The homologous series of fatty acids from C₆ to C₁₀ were run on the column to ensure that the octanoic

acid was completely resolved from any other acids on the column.

The total radioactivity of the synthesised acid was 0.0275 mc, approximately 14% of the initial radioactivity used (0.2 mc), which is in agreement with the percentage recovery obtained above.

Biosynthesis of 2-heptanone-1-C¹⁴. 25 ml of a solution of the potassium salt of 2-C¹⁴ octanoic acid (approximately 750 μ moles) were incubated with 80 ml potassium phosphate buffer, pH 6.0 (50 μ moles phosphate per ml) and 10 ml spore suspension (strain 6989, harvested 6 days after inoculation). Incubation was continued until the methyl ketone concentration reached a maximum. 50 ml of 2,4-DNP hydrazine reagent were then added and the mixture left for 24 hours. The 2,4-DNP hydrazone of 2-heptanone-1-C¹⁴ was extracted and purified as described in Part I. The weight of pure 2,4-DNP hydrazone was 0.205 g, the equivalent of 0.07 g 2-heptanone-1-C¹⁴, representing 68% conversion of acid to ketone.

The free 2-heptanone-1-C¹⁴ was liberated from the hydrazone with laevulinic acid as described in Part I. The solution of ketone in petroleum ether was evaporated carefully in a stream of air, the activity of the solution being measured during the evaporation with a portable transistorized monitor (Phillips type PW 4012). Evaporation was continued until the activity reached a maximum when it was assumed that all the petroleum ether had evaporated. The residue was shaken up in 100 ml water. 1 ml of this solution contained 1.95 μ moles heptanone, the percentage recovery of heptanone from its 2,4-DNP hydrazone thus being approximately 32%.

10 ml of the solution were extracted with 2 x 25 ml diethyl ether. The extract was dried with anhydrous Na_2SO_4 and evaporated down cautiously in air to 0.5 ml. This concentrated ethereal solution was shown to contain only 2-heptanone- 1-C^{14} by co-chromatography with a pure sample of 2-heptanone on a gas chromatograph using a column packing of 20% (w/w) carbowax 1546/chromosorb W (30-60 mesh).

Preparation of 2-heptanol. 2-heptanol was prepared by reducing 2-heptanone with sodium/ethanol. (Whitmore & Otterbacher, 1944). It was purified from unchanged 2-heptanone by shaking with 2,4-DNP hydrazine/HCl reagent and fractionally distilling under reduced pressure. This distillation was repeated 3 times before the alcohol was completely free of ketone, the purity being checked by gas chromatography using a 20% diethylene glycol succinate/chromosorb W (30-60 mesh) column.

EXPERIMENTAL CONDITIONS AFFECTING THE OXIDATION OF FATTY ACIDS

The effect of age of mycelium on oxygen uptake in presence of fatty acids

The optimum age of mycelium for maximum oxygen uptake and methyl ketone formation in the presence of fatty acids was shown to be between 48 and 60 hours. The oxidation of fatty acids by mycelium used when about 30 hours old was markedly slower. This confirms similar observations on oxygen uptake by fungi by Cochrane & Peck (1953) and Vinze & Ghosh (1961). It was also observed that mycelium older than 72 hours consistently gave low yields of methyl ketone.

Endogenous respiration of mycelium. The interpretation of oxygen uptake during the oxidation of added substrate is difficult since it has to be decided whether endogenous respiration is proceeding at the same time. In this study it was concluded that endogenous respiration was not suppressed during the exogenous respiration of non-toxic concentrations of fatty acids (in agreement with the findings of Blumenthal (1963) with other *Penicillia*) for the following reasons :

1. In the course of most experiments a change in the rate of oxygen consumption could be clearly shown and unambiguously interpreted as a transition from the breakdown of the substrate to respiration of cell materials (endogenous respiration). The rates of

oxygen uptake due to endogenous respiration before and after the addition of substrate were not significantly different.

(2) In many experiments quantitative balances were obtained by assuming that endogenous respiration was proceeding simultaneously and subtracting its value from the oxygen uptake in the presence of substrate.

(3) Oxidative assimilation of part of the substrate could not be demonstrated by the use of 2,4 dinitrophenol.

Oxidative assimilation of fatty acids by mycelium

2,4 dinitrophenol is known to prevent oxidative phosphorylation and therefore oxidative assimilation, i.e. the ability to convert a portion of the substrate into complex material within the cell. It was thought therefore that its addition in this investigation might enhance the complete oxidation of the octanoic acid by the mycelium. A range of concentrations of 2,4 dinitrophenol was tried but did not effect the oxygen uptake in any systematic way. The results in Table 12, using two different batches of mycelium and a final concentration in the reaction flask of 1×10^{-3} M 2,4 dinitrophenol were typical.

The effect of pre-starving mycelium in buffer

Although endogenous respiration of mycelium was not usually markedly decreased by shaking in buffer, pre-starving in buffer enabled the mycelium to oxidise

Table 12. The effect of 2,4 Dinitrophenol (2,4 DNP in this table) on the oxidation of octanoic acid by mycelium

Expt.	Concentration Octanoic acid	Oxygen Uptake (μ l)	2-heptanone (μ moles)
1	Endogenous	75	-
	1 μ mole	157	0.03
	1 μ mole + 10^{-3} M 2,4 DNP	160	0.05
	3 μ moles	184	0.4
	3 μ moles + 10^{-3} M 2,4 DNP	206	0.35
2	Endogenous	254	-
	1 μ mole	384	0.09
	1 μ mole + 10^{-3} M 2,4 DNP	332	0.12
	2 μ moles	420	0.53
	2 μ moles + 10^{-3} M 2,4 DNP	443	0.50

(Each Warburg contained 3 mg mycelium, 48 hours old, 50 μ moles phosphate pH 6.0; period of incubation 150 minutes).

Table 13. The effect of pre-starving mycelium in buffer on its ability to oxidise octanoic acid at pH 2.5

Concentration C ₈ acid	Oxygen Uptake (μl)		2-heptanone (μmoles)		
	Nil	1 μmole	3 μmoles	1 μmole	3 μmoles
Mycelium, fresh	155	285	22	0.45	0.32
" pre-starved 15 hrs	140	351	104	0.19	0.50
" " 20 "	145	350	150	0.14	0.70

(Each Warburg flask contained about 10 mg mycelium, grown for 65 hours, 50 μmoles phosphate pH 2.5 and distilled water to 3 ml. Period of incubation 120 minutes).

concentrations of fatty acid which inhibited the oxygen uptake of fresh mycelium (Table 13).

The oxygen uptake by pre-starved mycelium in the presence of non-toxic* concentrations of octanoic acid (1 μ mole) was also higher than with fresh mycelium but at the expense of methyl ketone formation.

Inter-relationship between pH and concentration of fatty acid substrate

At any one pH it was found that there was an optimum concentration of acid for maximum oxygen uptake and optimum conversion to methyl ketone. Fig. 6a shows the variation of oxygen uptake and methyl ketone formation with concentration of octanoic acid at pH 6.5, maximum uptake occurring with 3.5 μ moles. The results also show clearly that the toxic effect of octanoic acid at this pH becomes more pronounced with increasing substrate concentration.

The oxidation of any one concentration of fatty acid is dependent upon the pH of the incubation medium. Thus the results of Table 14 show that whereas 3 μ moles of

* Throughout this thesis the term "toxic" acid is defined as one that suppresses the oxygen uptake of mycelium relative to endogenous. It does not necessarily mean complete inhibition. Similarly a "non-toxic" acid is, in this context, one whose presence enhances or at least does not inhibit oxygen uptake by mycelium.

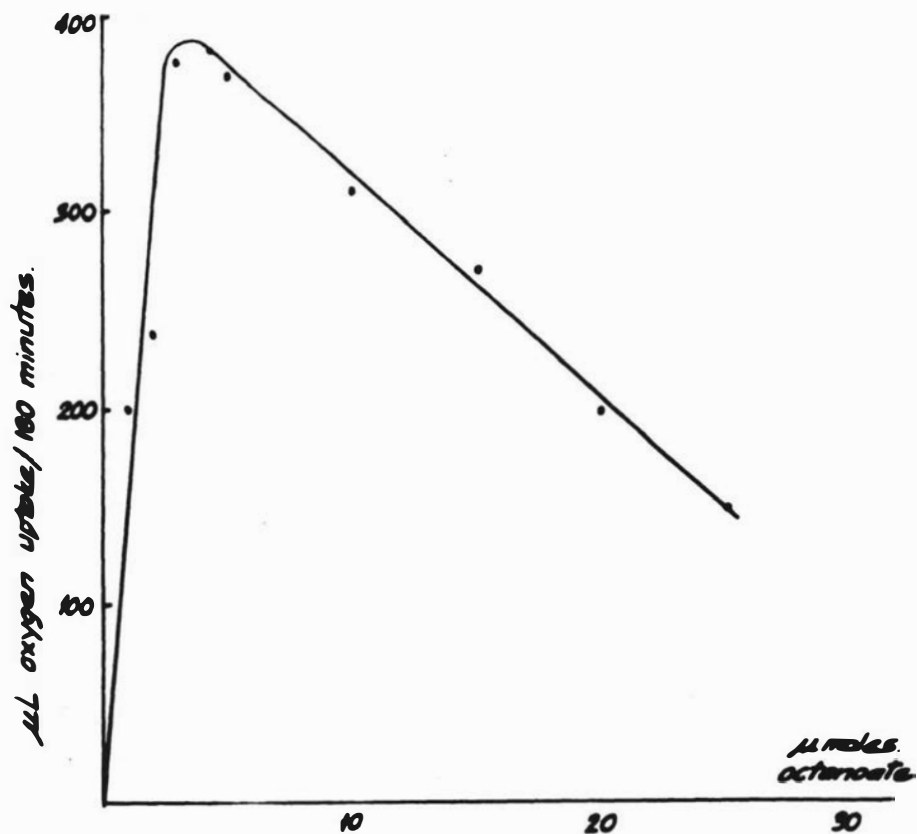


Fig.6a. Variation of oxygen uptake by mycelium with concentration of octanoic acid at pH 6.5. Each flask contained about 4 mg mycelium (dry weight) and 50 μ moles phosphate buffer.

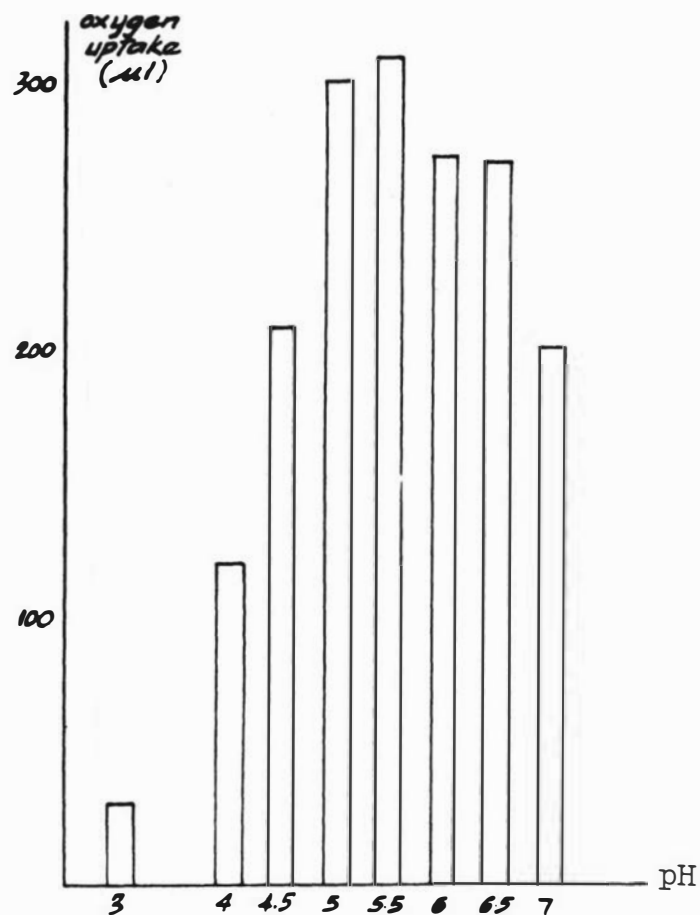


Fig.6b. The effect of pH on the oxygen uptake by mycelium in the presence of 3 μ moles octanoic acid. Each flask contained about 4 mg mycelium (dry weight) and 50 μ moles phosphate buffer. Period of incubation 180 min.

octanoic acid inhibited oxygen uptake at pH 2.6, forming little methyl ketone, the same concentration of acid at pH 5.0 and 6.3 was rapidly oxidised with high yields of 2-heptanone.

Oxygen uptake and methyl ketone formation during the oxidation of different concentrations of octanoic acid were obtained at various pH's in order to ascertain optimum conditions. With increasing concentration of octanoic acid, the pH at which oxygen uptake was a maximum also increased. Optimum pH's for the oxidation of 1, 3 and 25 μ moles of acid were pH 5.0, 5.5 and 6.0 respectively (Figs. 6b, 7 and 8). Similarly the formation of 2-heptanone from 1 μ mole of acid was a maximum at pH 3.0 (Fig. 7) but was optimum at pH 6.5 with 25 μ moles (Fig. 8). The correlation between pH and the concentration of the acid being oxidised is also illustrated in Table 14.

Effect of purity of acid

Gas chromatographic analysis showed that redistilled octanoic acid contained up to 10% butyric and hexanoic acids as impurities and that decanoic acid contained approximately 10% lauric acid. The impurities affected the oxygen uptake as the comparison between very pure octanoic (99.8 + %) and technical (BDH) octanoic acid showed (Table 15). This was confirmed by using equal amounts of both pure and technical acids in the Warburg flask when the oxygen uptake was intermediate between the two.

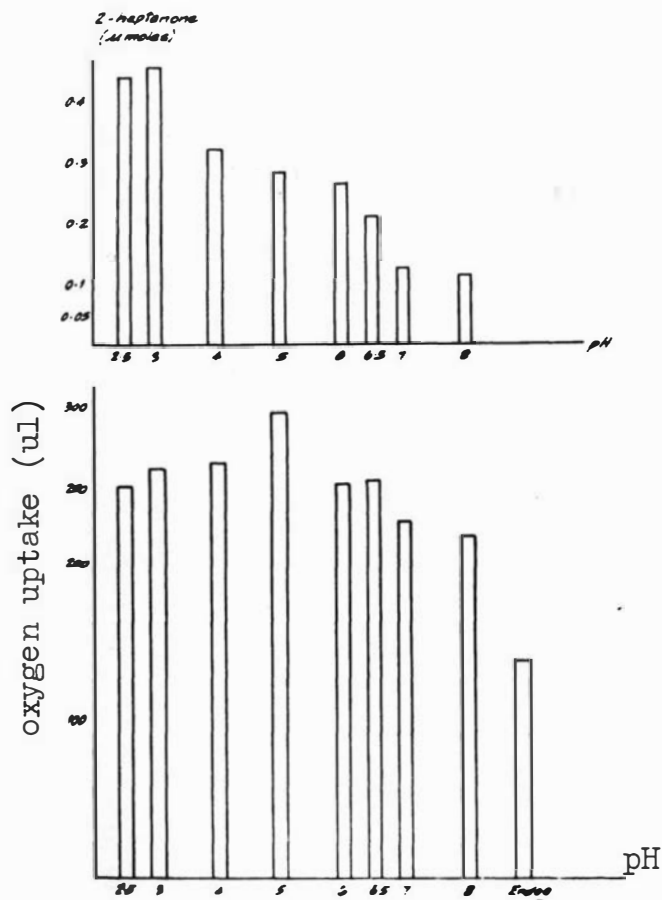


Fig.7. (1 μmole octanoic acid)

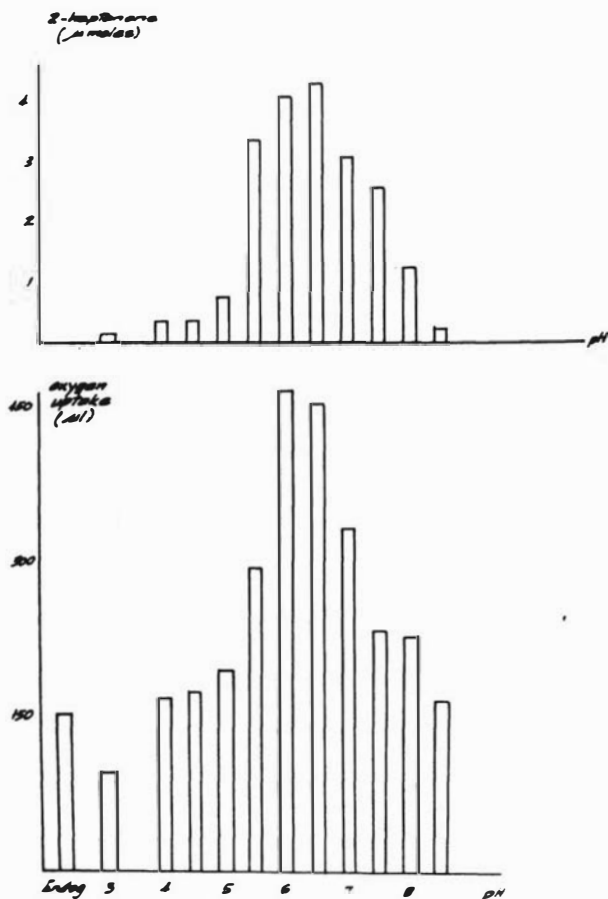


Fig.8. (25 μmoles octanoic acid)

The effect of pH on (a) oxygen uptake and (b) methyl ketone formation by mycelium in the presence of 1 μmole and 25 μmoles octanoic acid. (Figs. 7 and 8). Each flask contained about 4 mg mycelium, 50 μmoles phosphate buffer. Period of incubation 180 min.

Table 14. The combined effect of pH and concentration on the oxidation of octanoic acid by mycelium

pH	Oxygen Uptake (μ litres)			2-heptanone (μ moles)	
	Endogenous	3 μ moles acid	20 μ moles acid	3 μ moles acid	20 μ moles acid
2.6	335	202	34	0.32	nil
5.0	338	570	448	1.8	1.08
6.3	317	606	500	1.7	1.2
7.5	320	533	440	1.2	0.9

(Each Warburg contained approximately 10 mg. mycelium, 60 hours old, 50 μ moles phosphate buffer. Period of incubation 150 minutes).

Table 15. The effect of purity of octanoic acid on oxygen uptake by mycelium

pH	Oxygen Uptake (μ l)*	
	Pure acid	Technical acid
6	276	208
6.5	275	177
7	196	110

(Each Warburg flask contained approximately 3 mg mycelium, 48 hours old, 50 μ moles phosphate buffer, 3 μ moles octanoic acid. Period of incubation 180 minutes. * Endogenous respiration deducted).

Consequently pure fatty acids, obtained by means of a preparative gas chromatography column, were used in all but the initial stages of this investigation.

Effect of inorganic ions

(a) Magnesium and calcium ions. Since magnesium ion is reported as being generally required by oxidative systems (Lardy, 1951) its effect was given special attention. Stimulation of oxygen uptake was variable (Table 16) but tended to be slightly enhanced with decreased methyl ketone formation.

Similarly addition of calcium ion gave no consistent pattern of oxygen uptake and methyl ketone formation.

(b) Phosphate concentration. An optimum phosphate concentration for oxygen uptake by fresh mycelium was found to be about 10 μ moles/ml (Table 17), which confirms similar results obtained by Girolami & Knight (1955). Increasing phosphate concentration decreased oxygen uptake but increased the formation of methyl ketones. The complete absence of phosphate in the flask inhibited not only oxidation in the presence of acid but also the endogenous respiration.

Shaking the mycelium in phosphate buffer for 36 hours gave somewhat different results (Table 18), possibly a reflection of the observed decrease in toxicity of fatty acids towards pre-starved mycelium. Both oxygen uptake and methyl ketone formation reached a maximum at a phosphate concentration of 30-35 μ moles/ml, concentrations greater than this resulting in a lowering of both.

Table 16. The effect of magnesium ion on the oxidation of 1 μ mole octanoic acid

Magnesium Concentration (μ moles)	Oxygen Uptake (μ l)		2-heptanone (μ moles)
	Endogenous	Octanoic acid	
0	110	300	0.26
0.1	134	373	0.14
0.5	126	331	0.14
1.0	130	332	0.19

(Each Warburg contained approximately 10 mg mycelium, 62 hours old, 50 μ moles phosphate buffer, pH 5.5. Period of incubation 150 minutes).

Table 17. The effect of phosphate concentration upon oxygen uptake by fresh mycelium in the presence of octanoic acid (1 μ mole)

Phosphate buffer (μ moles), pH 6.0	Oxygen Uptake (μ l)		2-heptanone (μ moles)
	Endogenous	Octanoic acid	
0	74	61	nil
25	215	413	0.04
50	232	363	0.08
100	212	328	0.11
200	204	311	0.14

(Each Warburg flask contained 10 mg fresh mycelium, 60 hours old, Period of incubation 120 minutes).

Table 18. The effect of phosphate concentration upon the oxidation of octanoic acid (3 μ moles) by pre-starved mycelium

Phosphate buffer (μ moles), pH 6.0	Oxygen Uptake (μ l)		2-heptanone (μ moles)
	Endogenous	Octanoic acid	
0	-	90	nil
25	328	416	0.70
50	-	430	0.71
75	-	434	1.01
125	302	387	0.91
150	-	346	0.82
225	302	365	0.75

(Each Warburg contained 10 mg mycelium, grown for 60 hours and then shaken in buffer for 36 hours, Period of incubation 120 minutes).

Suitability of CO₂ formation as a measure of fatty acid oxidation

The method most widely used for determining respiration - the absorption of any CO₂ formed by alkali - is valid only if the cells respire at the same rate, to the same extent and follow the same pathways in the presence or absence of CO₂ (Dixon 1951). Since the formation of methyl ketones in the oxidation of fatty acids by the fungus almost certainly involved a decarboxylation, the effect of removing the carbon dioxide as it was formed was investigated.

Between pH 5.2 and 6.5 it was invariably found that the absence of KOH in the inner well of the Warburg flask increased the yield of methyl ketone, usually by about 5% when fresh mycelium was incubated with fatty acids but up to 50% with mycelium that had been pre-starved in buffer (Table 19). The effect was even more marked with spores, the formation of methyl ketones being enhanced by up to 100% (Table 20) when CO₂ was not absorbed.

The addition of comparatively large amounts of bicarbonate (10-100 μmoles) however reduced ketone formation almost completely. Neither was ketone detected when the spores were incubated in either an atmosphere of carbon dioxide or of nitrogen.

Table 19. Effect of absorbing CO₂ on the formation of methyl ketones from fatty acids by mycelium pre-starved for 12 hours in buffer

Fatty Acid (3 μ moles)	Methyl Ketone (μ moles)	
	+ KOH	- KOH
Hexanoic	1.30	1.41
Octanoic	1.56	2.30

(Each Warburg flask contained 10 mg pre-starved mycelium, 48 hours old, 50 μ moles phosphate, pH 6.0. Period of incubation 180 minutes).

Table 20. The effect of absorbing CO₂ on the formation of methyl ketones from fatty acids by spores

Fatty acid (3 μmoles)	Methyl Ketones (μmoles)			
	6 hours		20 hours	
	+ KOH	- KOH	+ KOH	- KOH
C ₄	Nil	Nil	Nil	Nil
C ₆	Nil	0.10	Nil	0.10
C ₈	0.70	0.95	1.40	1.80
C ₁₀	0.15	0.29	0.21	0.56
C ₁₂	0.10	0.26	0.11	0.27
C ₁₄	Nil	Nil	Nil	Nil

(Each Warburg flask contained 1×10^7 spores, 4 days old, 50 μmoles phosphate buffer pH 6.25).

Oxygen balances for the oxidation of octanoic acid by mycelium

Theoretically the complete oxidation of 1 μ mole octanoic acid to CO_2 and H_2O needs 11 μ moles oxygen (246 μ l). If allowance were made for the considerable amounts of 2-heptanone formed (1 μ mole octanoic acid needs 1 μ mole oxygen, i.e. 22.4 μ l, for complete oxidation to 2-heptanone) oxygen uptakes were almost quantitative (Table 21).

Comparative rates of formation of methyl ketones by mycelium and spores

In view of the claim by Gehrig & Knight (1963) that the spores of P. roqueforti and not the mycelial cells were responsible for formation of methyl ketones from fatty acids it was of interest to compare the rates of formation of methyl ketone by both mycelium and spores.

Mycelium not containing spores was obtained by blending every 24 hours and re-inoculating in fresh medium, any spores enveloped initially in the medium being released by the blending. A portion of the same batch of mycelium was also pre-starved in phosphate buffer, pH 5.2, for 24 hours. Fresh and pre-starved mycelium, and also 4 day old and 9 day old spores, were incubated with octanoic acid (3 μ moles), the flasks being analysed for methyl ketones at hourly intervals. The pattern of oxidation was almost the same (compare Figures 10a & 10b), a lag period of approximately 1 hour

Table 21. Oxygen balances obtained during the oxidation of octanoic acid by different batches of mycelium

Concentration octanoic acid (μ moles)	pH	2-heptanone (μ moles)	Oxygen Uptake (μ litres)		
			Observed	Theoretical	$\frac{\% \text{ Observed}}{\text{Theoretical}}$
1	6.2	0.18	194	205	95
1	6.2	0.26	190	188	101
2	6.3	0.70	341	331	103
2	6.2	0.79	279	297	94
2	6.0	0.80	300	295	102
3	5.5	1.70	312	320	98
3	5.2	1.61	272	302	91
1	2.9	0.45	130	145	90
1	2.9	0.19	211	199	103

(Each flask contained approximately 10 mg mycelium, 50 μ moles phosphate buffer and distilled water to 3 ml. Oxygen uptake was measured until all substrate had been oxidised after 2 to 3 hours incubation).

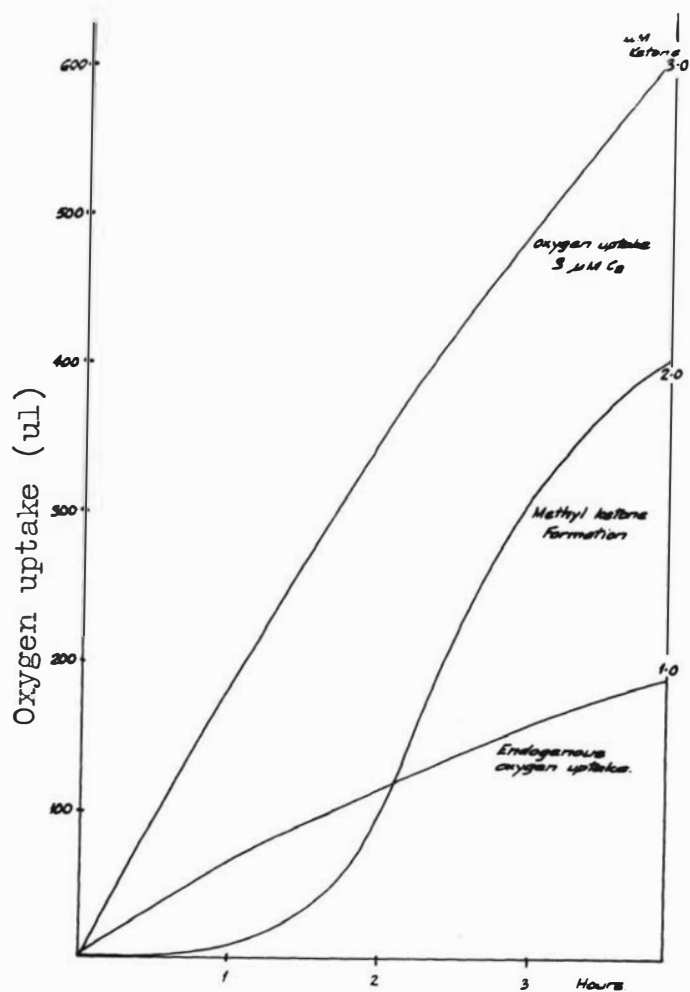


Fig. 10a.

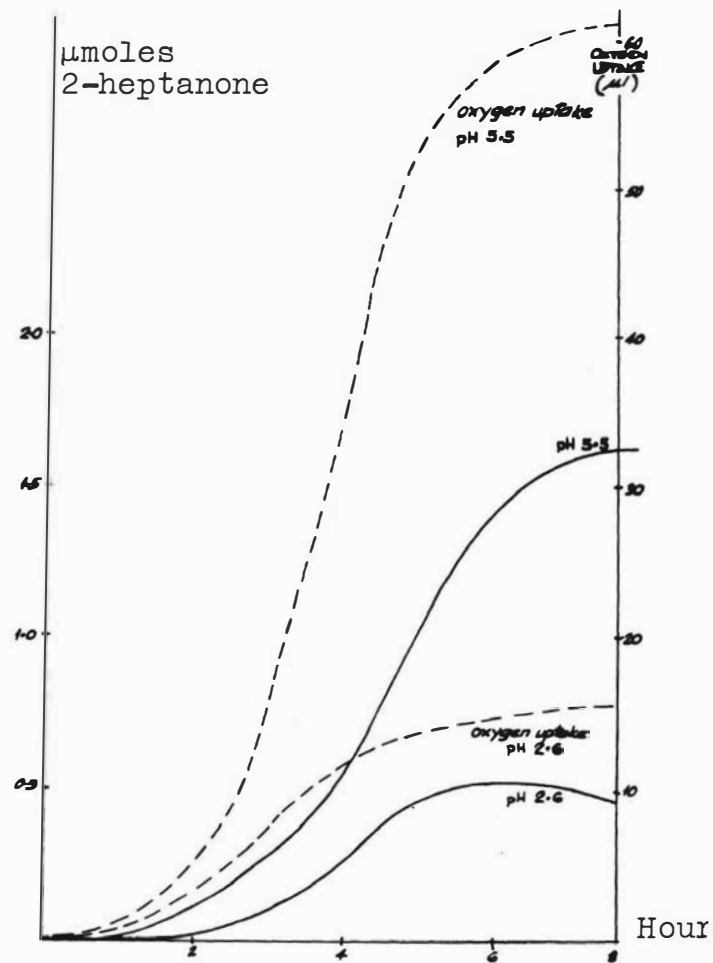


Fig. 10b.

Rates of oxygen uptake and methyl ketone formation by mycelium (Fig.10a) at pH 5.2, and by spores (Fig.10b) at pH 2.5 and 5.5, in the presence of 3 μmoles octanoic acid and 50 μmoles phosphate buffer.

occurring in each case before methyl ketone formation was detected. Maximum ketone formation was observed after 3 to 4 hours and then slowly decreased. Both fresh and pre-starved mycelium formed more methyl ketone than the spores (Table 22), although the reverse was more usual.

Relationship between methyl ketone formation and growth medium of spores

The cultural background of spores have a marked influence on their enzyme pattern and physiological properties (Darby & Mandels, 1955; McCormick & Halvorson, 1963). The ability of spores to oxidise fatty acids to the corresponding methyl ketone however was not significantly dependent in the present study upon the medium (Czepek-Dox, potato-dextrose and malt agars were tested) on which the spores were grown.

The effect of age on the ability of spores to metabolise fatty acids

At pH 5.2, 4 day old spores oxidised about 40% and 9 day old spores about 50% of the octanoic acid (3 μ moles) to 2-heptanone. A lag of 1 hour occurred before any ketone was detected with 4 day old spores and one of about 2 hours for the 9 day old spores (Fig. 9). The yield of ketone reached a maximum after 6 hours for 4 day old spores and after 7 hours for 9 day old spores. Between the 7th and 21st hours of incubation,

Table 22. Comparison of ability of fresh and pre-starved mycelium and spores to form 2-heptanone from octanoic acid (3 μ moles)

Time (hours)	Fresh Mycelium	Pre-starved Mycelium	4 day old spores	9 day old spores
3	1.20	1.72	0.96	0.12
4	1.60	1.70	1.10	0.41
6	1.58	1.52	1.19	1.36

(Each Warburg flask contained either 10 mg mycelium, 60 hours old, or 1×10^7 spores, 4 or 9 days old; 50 μ moles phosphate buffer pH 5.2).

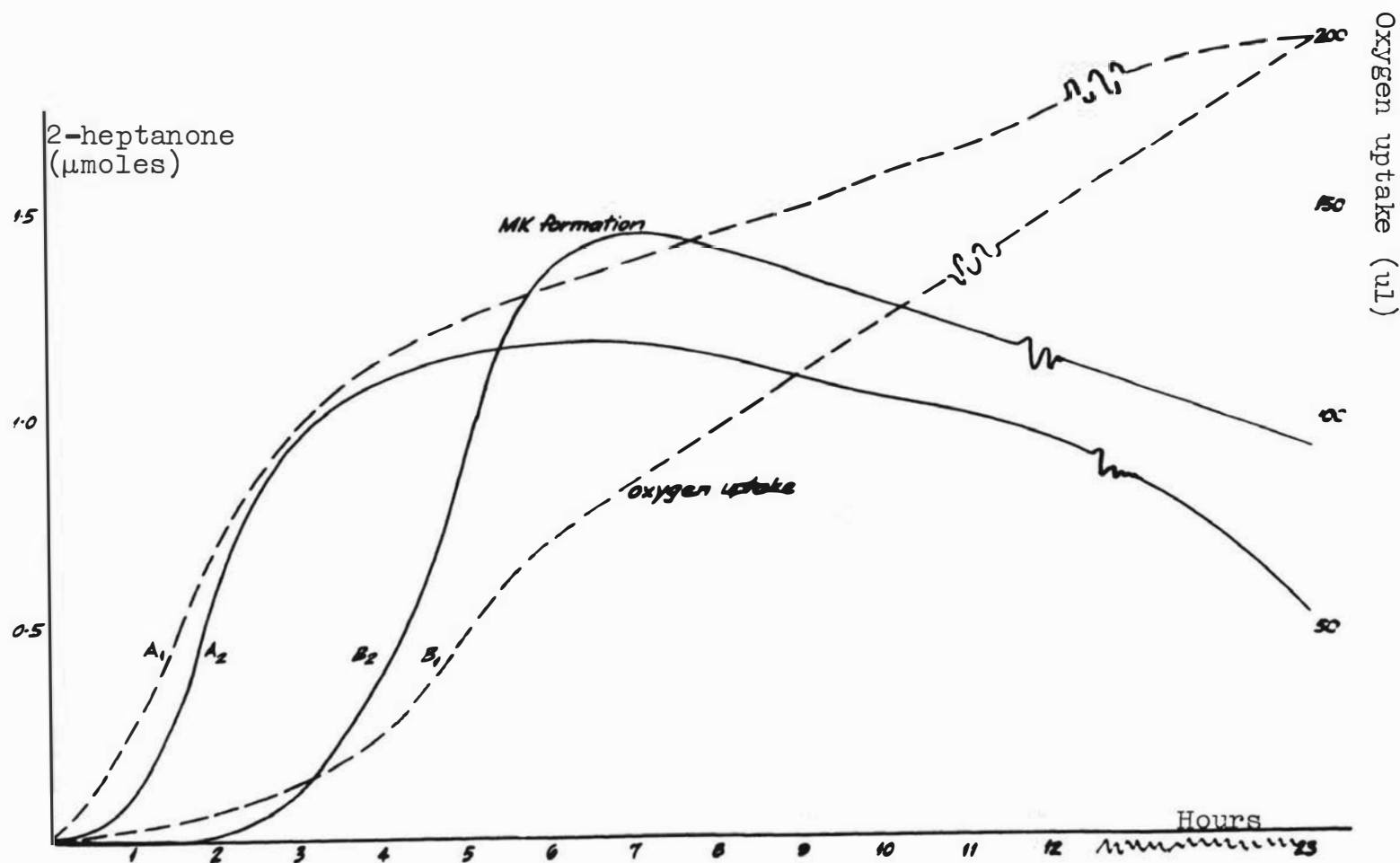


Fig.9. Oxygen uptake and methyl ketone formation by 4 day old spores (Curves A₁ and A₂ respectively) and 9 day old spores (Curves B₁ and B₂) in the presence of 3 μmoles octanoic acid and 50 μmoles phosphate buffer, pH 5.2.

0.42 μ moles of 2-heptanone were metabolised by the 4 day old spores. The observed oxygen uptake in excess of the endogenous respiration over the same period was 112 μ l whereas theoretically 96 μ l would be needed for the complete oxidation of the 2-heptanone to CO_2 and H_2O . The 9 day old spores metabolised the 2-heptanone at about the same rate as the younger spores.

At pH 6.2 the oxygen uptakes during the oxidation of fatty acids were about 3 times greater with 4 day old than with 9 day old spores. The latter however were significantly more efficient than the young spores in oxidising C_{10} and C_{12} acids to the corresponding methyl ketones (Table 23). In general, older spores (9-14 days) formed greater amounts of methyl ketone than younger spores and were therefore used in most experiments.

Metabolism of octanoic acid over long periods

0.1% octanoic acid (approximately 9.5 μ moles/ml) in 40 ml of phosphate buffer, pH 6.0, was incubated at 22° with sporeless mycelium, 72 hours old. As Fig. 11 shows, the yield of ketone reached a maximum after 1½ days (when 36% of the acid had been oxidised to 2-heptanone) and thereafter was metabolised slowly but completely after 10 days. The very slow rate of metabolism of methyl ketone established that the percentage lost by further metabolism in respiration experiments was not significant. As long as fatty acid was available, it appeared that any methyl ketone formed

Table 23. Amounts of methyl ketone formed during the oxidation of various fatty acids by 4 and 9 day old spores

Fatty Acid (3 μ moles)	Methyl Ketone (μ moles)	
	4 day old spores	9 day old spores
C ₈	2.30	2.02
C ₁₀	0.63	1.40
C ₁₂	0.27	0.56
C ₁₄	Nil	Nil

(Each Warburg flask contained 1×10^7 spores, 50 μ moles phosphate buffer pH 6.2. Period of incubation 6 hours).

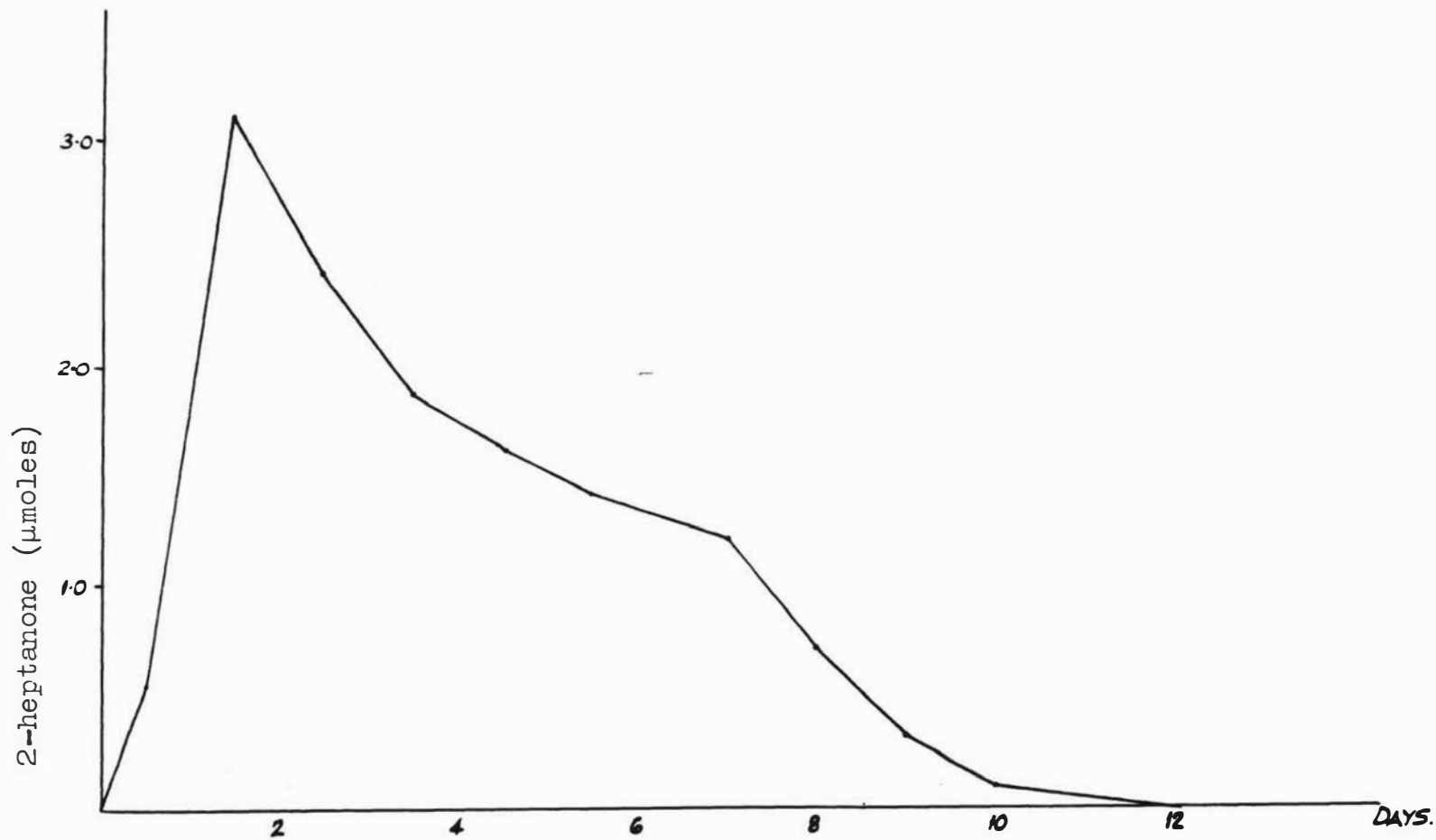


Fig.11. The formation of 2-heptanone from octanoic acid (8.5 μmoles/ml) by mycelium, 72 hours old, at pH 6.0 (50 μmoles phosphate buffer/ml).

as a by-product was not utilised by the fungus.

The same experiment was carried out with spores instead of mycelium. The relatively high concentration of acid was only slowly oxidised, a maximum yield (10%) of 2-heptanone being reached after 35 days (Fig. 12). It should be noted that there was no correlation in this instance between age of spores and the formation of 2-heptanone from octanoic acid.

Effect of malonate and diethyl malonate on the oxidation of fatty acids by mycelium

It was expected that the addition of malonate, a competitive inhibitor of succinate oxidation and thus of the TCA cycle, would result in an accumulation of β -keto acid and thereby in an increase in yield of methyl ketone. However mycelium was not only able to oxidise fatty acids readily in the presence of up to 100 μ moles of malonate per 3 ml medium but also malonate itself at pH's > 5.0 .

Since it was considered that the lack of inhibition at these pH's might be due to the absence of a sufficient concentration of undissociated molecules in the cells, the malonate was replaced by diethylmalonate. Its effect was to suppress the endogenous respiration of fresh mycelium and also both oxygen uptake and methyl ketone formation in the presence of fatty acids (Table 24). Diethyl malonate was inhibitory only below pH 5.5, in reasonable agreement with the conclusion of Bier (1955) that esters of dicarboxylic acids were not hydrolysed above a pH of about 5.0.

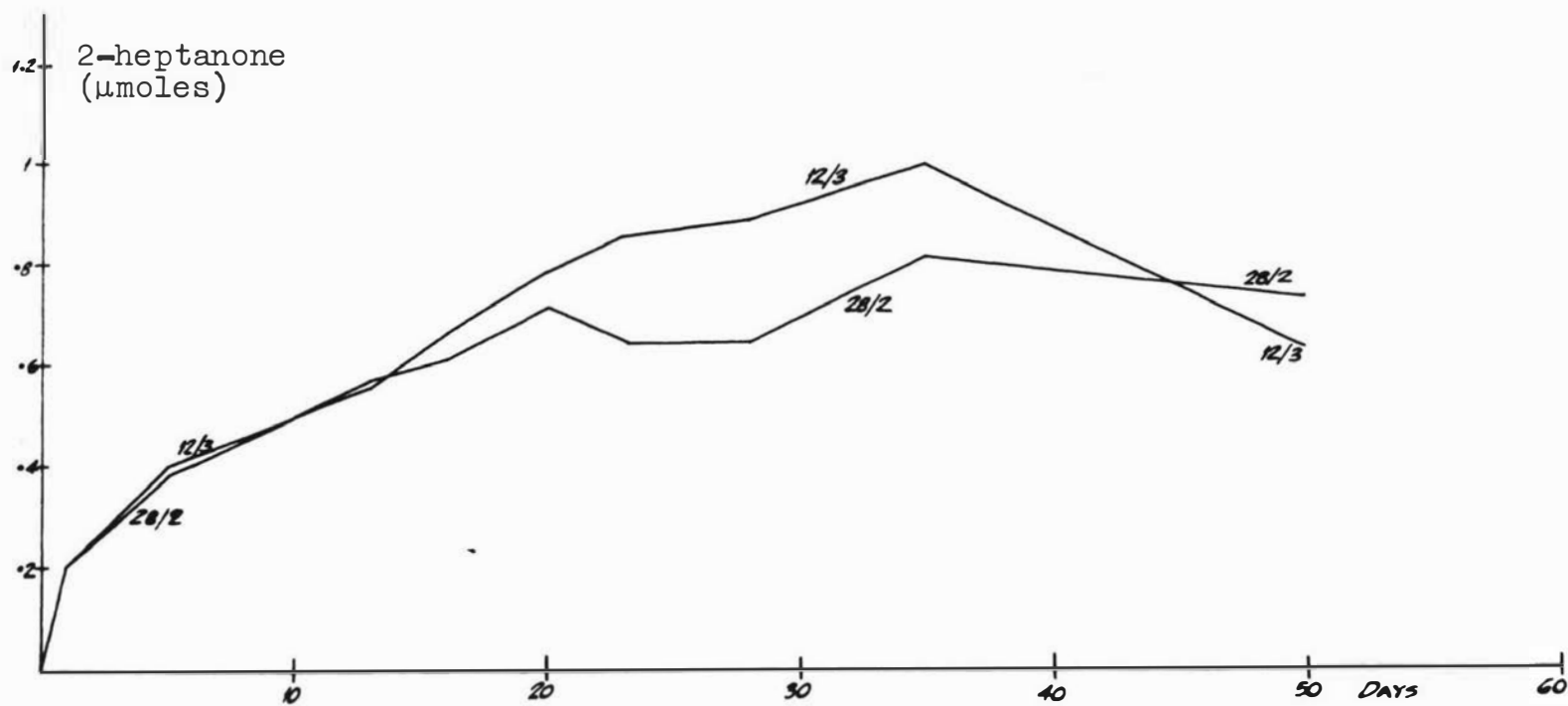


Fig.12. The formation of 2-heptanone from octanoic acid (8.5 μmoles/ml) by 6 and 18 day old spores (labelled 12/3 and 28/2 respectively above) at pH 6.0 (50 μmoles phosphate buffer/ml).

Table 24. The effect of diethylmalonate (DEM) on the oxidation of fatty acids by fresh mycelium.

	Oxygen Uptake (μ l)	Methyl Ketone (μ moles)
Mycelium, endogenous	525	-
" + 100 μ moles DEM	141	-
" + 4 μ moles C ₁₀ acid	82	0.04
" " " + 100 μ moles DEM	Nil	Nil
" + 3 μ moles C ₈ acid	694	1.25
" " " + 100 μ moles DEM	185	0.16

(Each Warburg flask contained approximately 10 mg mycelium, 4.2 hours old, 50 μ moles phosphate buffer pH 5.2. Period of incubation 180 minutes).

SUMMARY OF PRELIMINARY EXPERIMENTS

1. The endogenous respiration of mycelium appeared to proceed simultaneously with the oxidation of added fatty acid (in non-toxic concentrations).
2. Concentrations of fatty acid that inhibited oxygen uptake by fresh mycelium were usually readily oxidised by mycelium that had been pre-starved in buffer.
3. The ability to form methyl ketones from fatty acids was an intrinsic property of both spores and mycelium. 48 to 60 hours old mycelium and spores about 14 days old formed maximum amounts of methyl ketones.
4. Metabolism of the C₆ to C₁₀ fatty acids led to the formation of the corresponding methyl ketone with one less carbon atom. Thus only 2-pentanone was formed from hexanoic acid, 2-heptanone from octanoic acid and so forth.
5. The metabolism of 2-heptanone was slow and not significant in short term experiments (up to 3 hours).
6. At any one pH there was an optimum concentration of fatty acid for maximum oxygen uptake and formation of methyl ketone.
7. The ability of octanoic acid at any one pH to inhibit mycelial respiration became more pronounced with increasing substrate concentration.
8. The absence of KOH in the inner cell of the Warburg flasks invariably increased the yield of methyl ketones from fatty acid by both spores and mycelium. It was

not valid therefore to measure the CO_2 evolved by the "direct" manometric technique of comparing oxygen uptake in the presence and absence of KOH.

9. If allowance were made for the considerable amounts of 2-heptanone formed, oxygen balances for the oxidation of octanoic acid by mycelium were almost quantitative.

10. Oxidative assimilation appeared not to be of importance in the oxidation of fatty acids by mycelium.

11. Both oxygen uptake and methyl ketone formation from fatty acids were inhibited by the addition of diethyl malonate at pH's below 5.5.

OXIDATION OF FATTY ACIDS WITH AN EVEN NUMBER OF CARBON
ATOMS FROM C₄ TO C₁₈ BY MYCELIUM

Using the optimum conditions determined above, the rate of oxidation of a range of fatty acids (1 μ mole/ml) by mycelium (fresh and pre-starved) was investigated.

Oxidation of fatty acids at pH 2.5

Increase in oxygen uptake over endogenous respiration by fresh mycelium in the presence of fatty acid substrate was small and limited to a few acids. Maximum increase was obtained with C₄ acid, the most toxic acid being C₁₀ (Fig. 13). Methyl ketone formation from C₆ and C₈ acids was high even although both acids partially inhibited endogenous respiration. The C₁₂ and C₁₄ acids formed a small amount of ketone at this pH but subsequent analysis showed that this was a mixture of acetone and 2-undecanone for C₁₂ acid and that only acetone was formed in the oxidation of C₁₄ acid.

Shaking the mycelium in buffer for 48 hours considerably increased the ability of the mycelium to subsequently oxidise added fatty acids, particularly the C₄ - C₁₂ acids. Thus whereas the C₆ to C₁₂ acids suppressed the uptake of oxygen by fresh mycelium, C₆ was rapidly oxidised by pre-starved mycelium and the C₈ - C₁₂ acids were considerably less toxic.

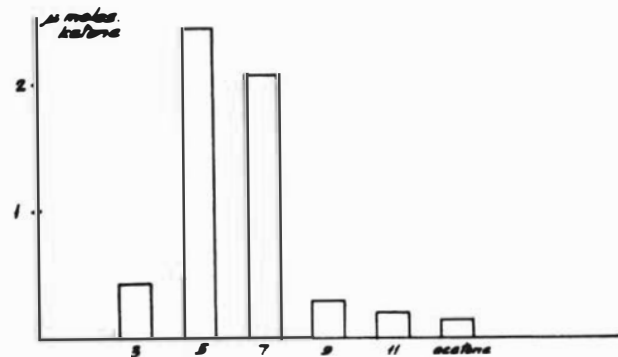
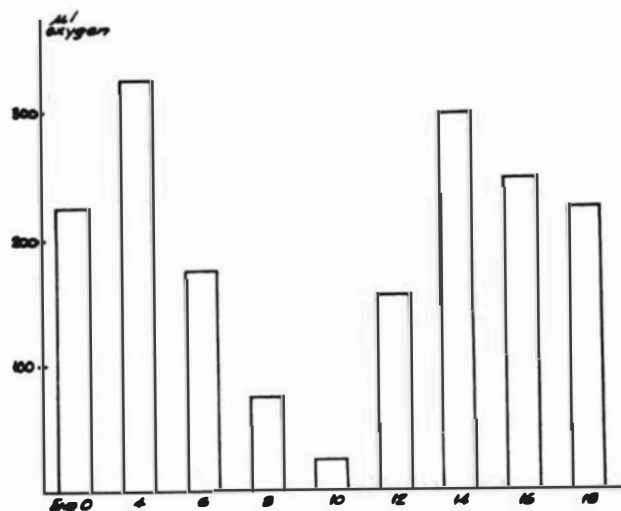


Fig.13.

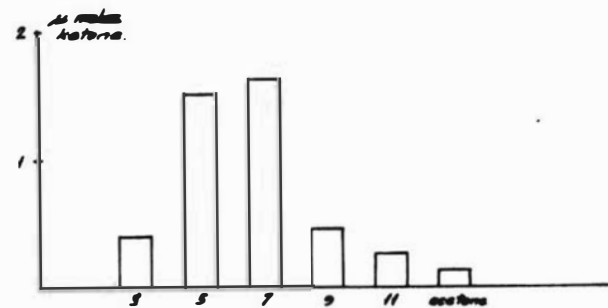
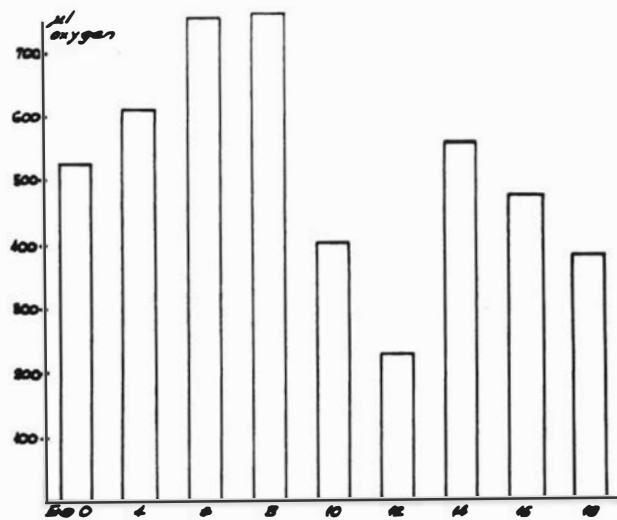


Fig.14.

Relationship between the chain length of "even-numbered" fatty acids and (a) the oxygen uptake and (b) methyl ketone formation by mycelium (66 hours old) at pH 2.5 (Fig.13) and at pH 5.2 (Fig.14). Each flask contained about 10 mg mycelium (dry weight), 3 μ moles of acid, 50 μ moles phosphate buffer and distilled water to 3 ml.

Oxidation of fatty acids at pH 5.2

The pattern of oxygen uptake (Fig. 14) was essentially the same as at pH 2.5 except that C₁₂ acid was the most toxic of the acids and that C₁₀ acid was less toxic than previously. Oxidation of C₆ and C₈ acids again gave the maximum yields of the corresponding methyl ketone, small amounts only being formed from the other acids up to C₁₄. Pre-starving the mycelium in buffer for 48 hours decreased the toxic action of all acids uniformly, the pattern of oxygen uptake remaining the same as with fresh mycelium.

Oxidation of fatty acids at pH 6.0

With fresh mycelium both oxygen uptake and ketone formation were a maximum when C₈ acid was used as substrate (Fig. 15). C₁₀ acid no longer inhibited oxygen uptake, the rate of uptake being greater for C₁₀ than for the C₁₂ and higher acids. Formation of 2-pentanone from C₆ acid was considerably lower at pH 6.0 than at pH 2.5 or 5.0. No corresponding methyl ketones were formed using C₁₄ to C₁₈ as substrates but very small amounts of acetone were detected.

The inhibition of oxygen uptake by fresh mycelium in the presence of C₁₂ and C₁₄ acids was overcome by shaking the mycelium in pH 6.0 buffer for 30 hours, although C₁₂ acid remained the least readily oxidised of the acids. The pattern of formation of methyl ketone was unaltered by pre-starving the mycelium in buffer.

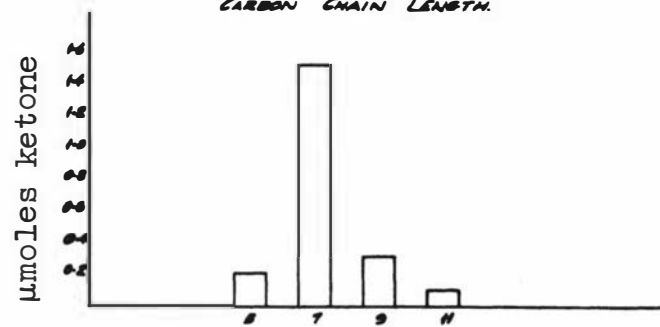
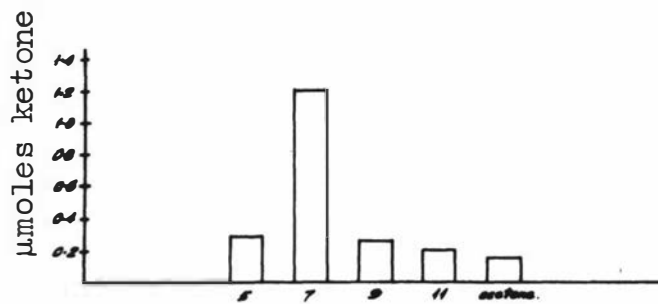
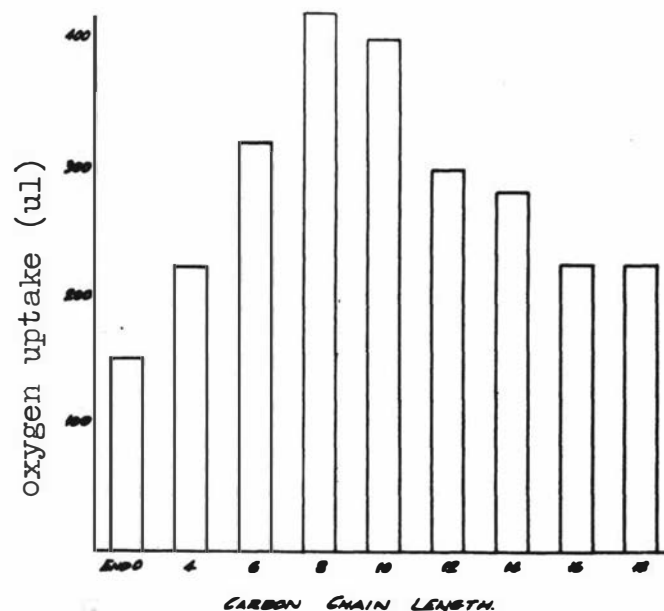
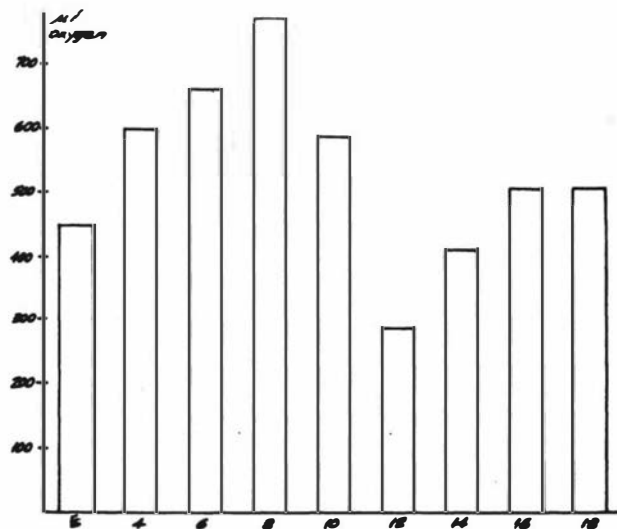


Fig.15.

Fig.16.

Relationship between the chain length of "even-numbered" fatty acids and (a) the oxygen uptake and (b) methyl ketone formation by mycelium (66 hours old) at pH 6.0 (Fig.15) and at pH 6.8 (Fig.16). Each flask contained about 10 mg mycelium (dry weight), 3 μ moles of acid, 50 μ moles phosphate buffer and distilled water to 3 ml.

Oxidation of fatty acids at pH 6.8

All fatty acids tested were oxidised by fresh mycelium, maximum oxygen uptakes being obtained with C₈ and C₁₀ acids as substrates. The yields of corresponding methyl ketone were similar to those found at pH 6.0 (Fig. 16). As at other pH's however the higher fatty acids (C₁₄ - C₁₈) were not oxidised to any significant extent, either by fresh or by pre-starved mycelium. Contrary to the findings of Rolinson (1954) and Vinze & Ghosh (1962), increasing the concentration of C₁₆ acid from 3 umoles to 20 umoles did not increase the oxygen uptake.

Oxidation of fatty acids at pH 8.0

Increase of oxygen uptake over endogenous respiration was very small except when using C₈ and C₁₀ acids as substrates (Fig. 17). Rates were lowest for the higher fatty acids (C₁₄ - C₁₈), C₁₄ acid actually inhibiting oxygen uptake by fresh mycelium. Yields of the corresponding methyl ketones were also low, being most readily formed with C₁₀ acid as substrate.

Oxidation of oleic acid

Oleic acid was readily oxidised in the pH range 2.5 to 8.0 (Figs. 18-20) although no methyl ketone formation was detected.

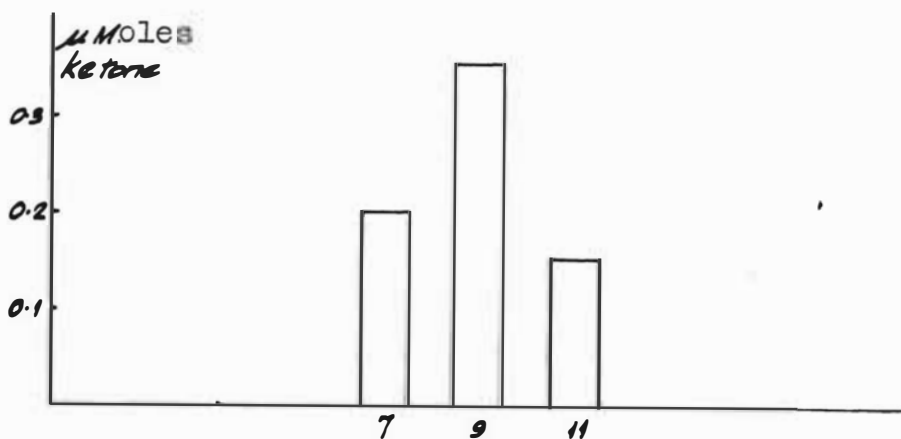
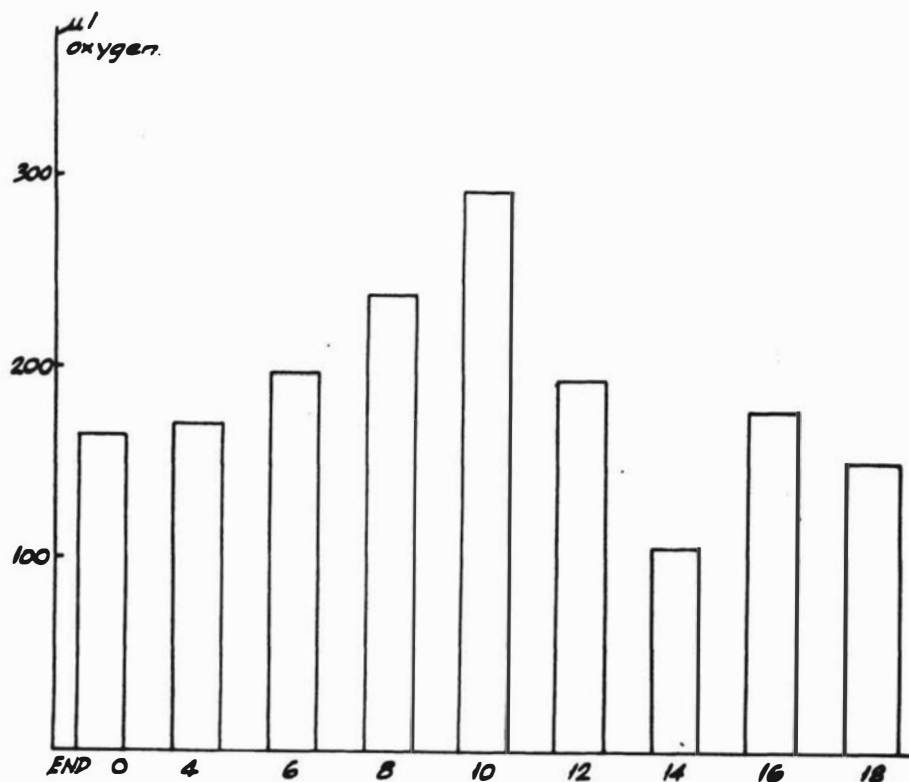


Fig. 17. Relationship between the chain length of "even-numbered" fatty acids and (a) the oxygen uptake and (b) methyl ketone formation by mycelium (66 hours old) at pH.8.0. Each flask contained about 10 mg mycelium (dry weight) 3 μmoles of acid, 50 μmoles phosphate buffer, and distilled water to 3 ml.

OXIDATION OF FATTY ACIDS WITH AN ODD NUMBER OF CARBON ATOMS BY MYCELIUM

The pattern of oxygen uptake and of methyl ketone formation was very similar to that obtained with fatty acids containing an even number of carbon atoms, suggesting that the oxidative pathway for all fatty acids is similar. Figures 18 - 20 show the rate of oxidation of the acids at different pH's. A number of acids with an even number of carbon atoms were included to enable comparison to be made with the previous experiments.

At pH 2.5 the oxygen uptake increased with the number of carbon atoms in the molecule from C₁ to C₅ acids. The higher fatty acids from C₇ to C₉ acids suppressed the uptake of oxygen by fresh mycelium (Fig. 18). The extent of oxygen uptake over endogenous respiration using C₁ and C₃ acids as substrates was sufficient to account for the complete oxidation of the acids to CO₂ and H₂O. A quantitative oxygen balance was obtained for C₅ acid when the yield of 2-butanone was also taken into account. The pattern of methyl ketone formation was very similar (Fig. 21 A) to that from "even-numbered" fatty acids at pH 2.5.

At pH 5.2 rates of oxygen uptake were highest when C₅ and C₇ acids were used as substrates (Fig. 19). C₉, C₁₀ and C₁₁ acids inhibited oxygen uptake by fresh mycelium and formed only small amounts of methyl ketone (Fig. 21B). The greatest yield of methyl ketone was obtained from C₅.

At pH 8.0 a similar pattern was found as for the oxidation of acids with an even number of carbon atoms. C₃ and C₅ acids were barely oxidised whereas C₉, C₁₀ and C₁₁ acids were those most readily oxidised (Fig. 20).

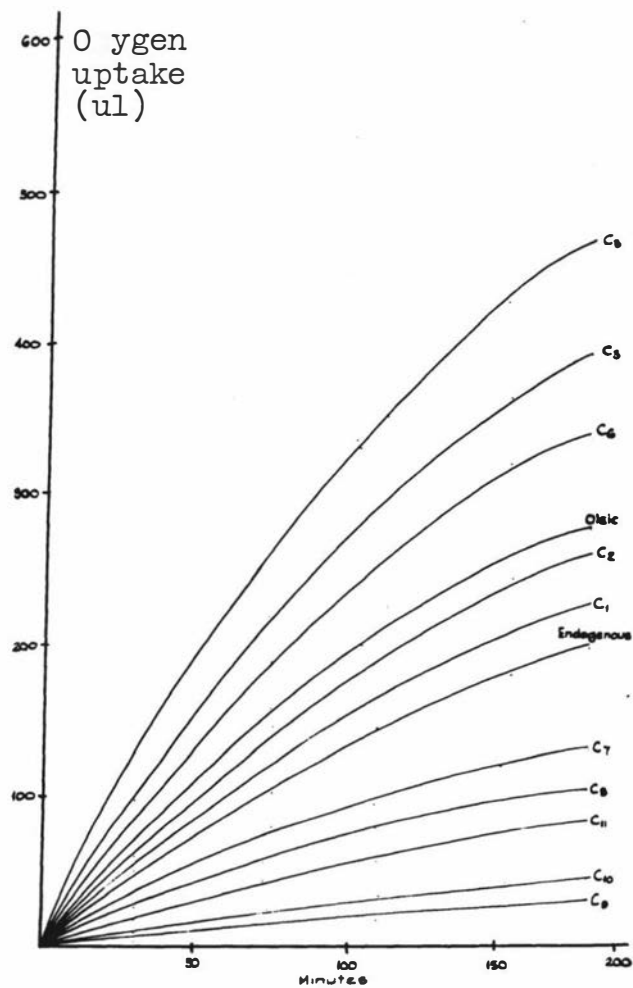


Fig.18.

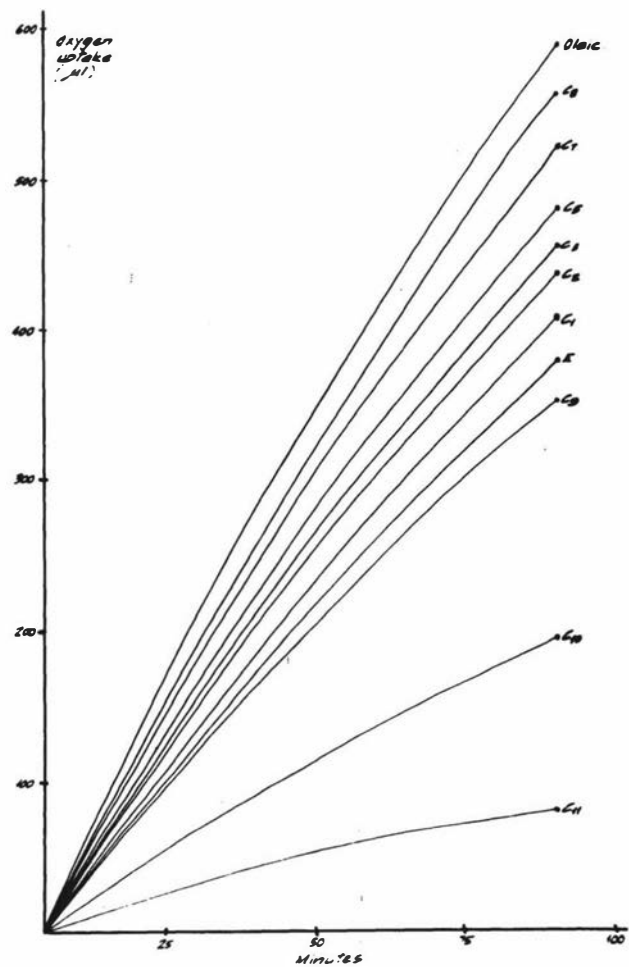


Fig.19.

Relationship between the chain length of "odd-numbered" fatty acids and oxygen uptake by mycelium (66 hours old) at pH 2.5 (Fig.18) and at pH 5.2 (Fig.19). Each flask contained about 10 mg mycelium (dry weight), 3 μ moles of acid and 50 μ moles phosphate buffer. E = endogenous respiration.

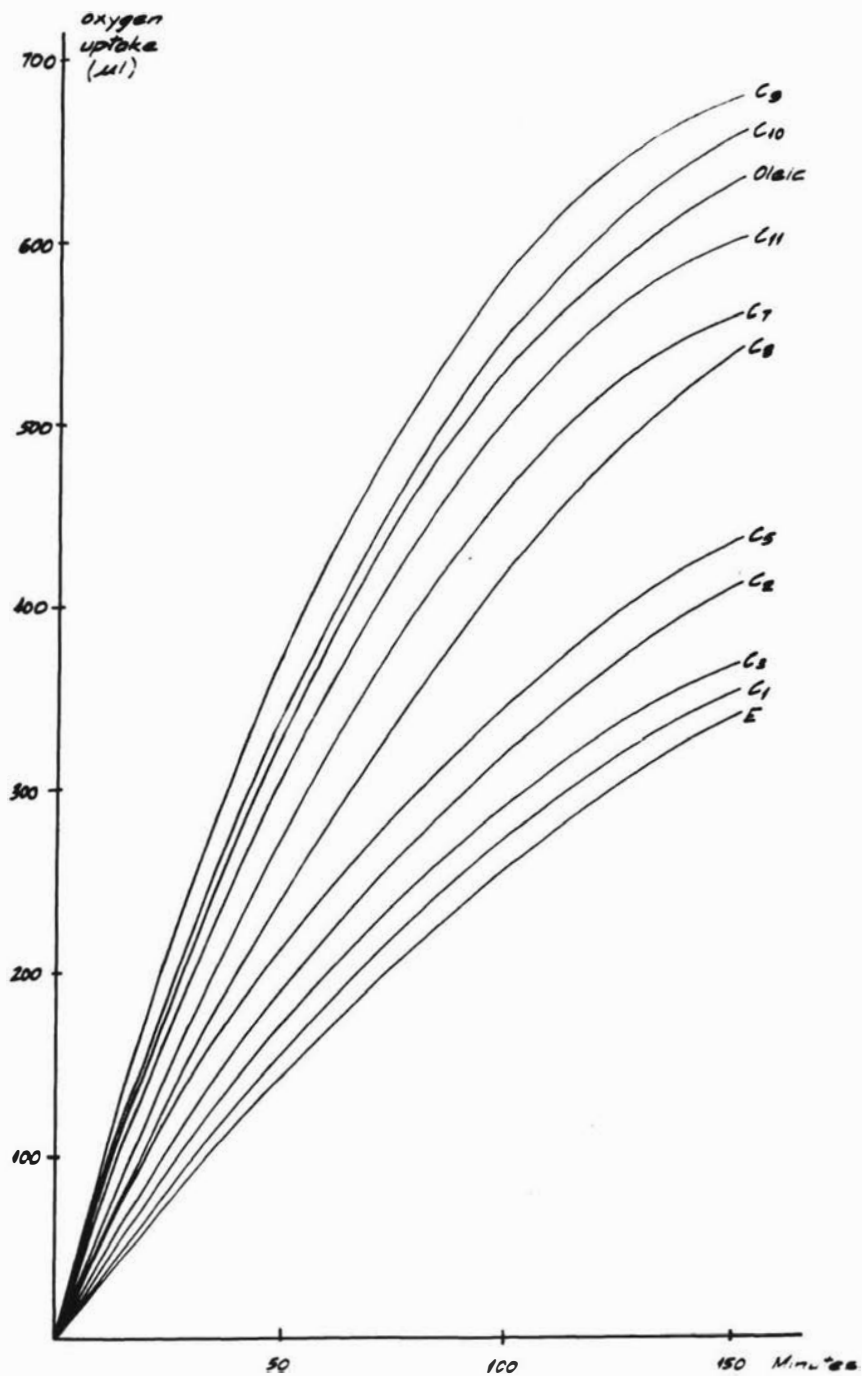
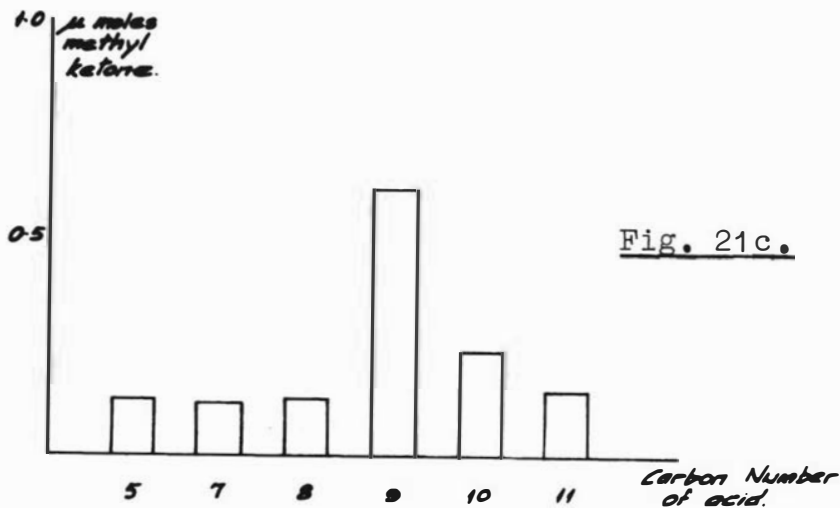
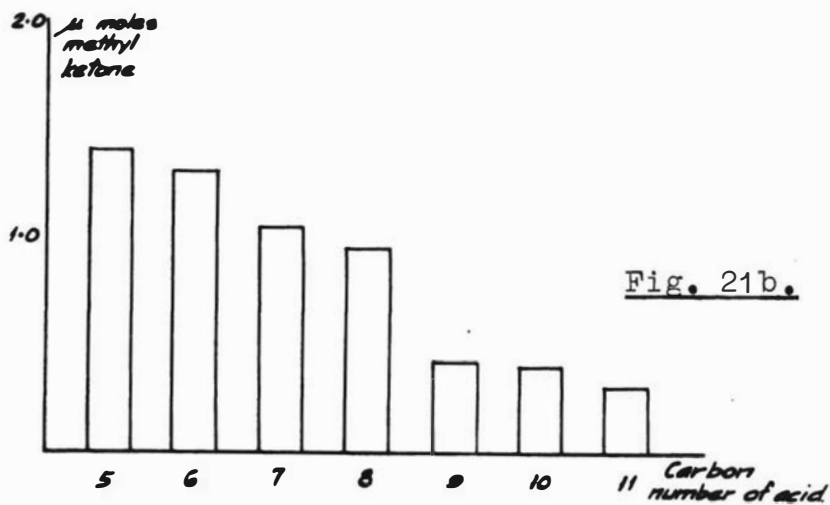
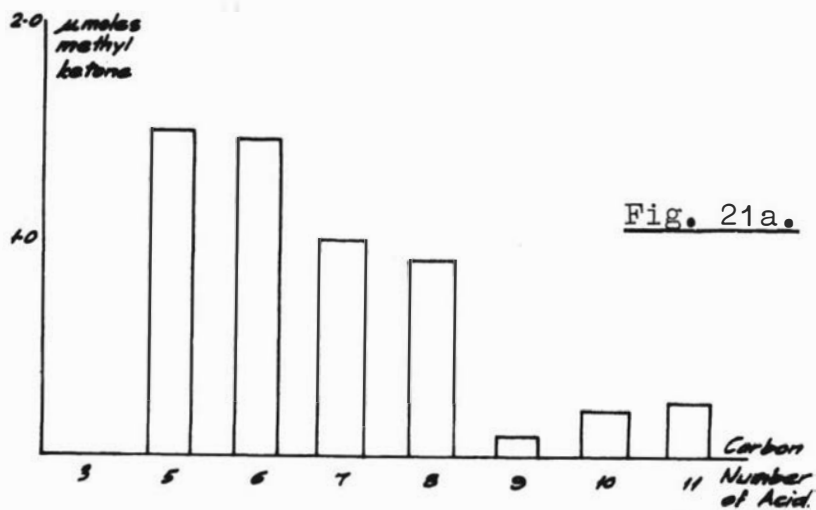


Fig.20.

Relationship between the chain length of "odd-numbered" fatty acids and oxygen uptake by mycelium (66 hours old) at pH 8.0. Each flask contained about 10 mg mycelium (dry weight), 3 μ moles of acid and 50 μ moles phosphate buffer. E = Endogenous respiration.



Relationship between chain length of "odd-numbered" fatty acids and methyl ketone formation by mycelium at pH 2.5 (Fig.21a), pH 5.2 (Fig.21b), and pH 8.0 (Fig.21c). Conditions otherwise as for Fig.20.

The greatest yield of the corresponding methyl ketone was obtained from C₉ acid (Fig. 21C).

OXIDATION OF FATTY ACIDS BY SPORES

The rate of oxygen uptake and the formation of methyl ketones by spores followed the same pattern of dependency upon pH as did mycelium. At pH 2.6 with octanoic acid as substrate the uptake of oxygen by 4 day old spores was low in comparison to that at pH 5.5 (Fig. 10B). Similarly the uptake of oxygen by 9 day old spores at pH 5.2 was low using C₁₀ and C₁₂ acids as substrate whereas oxygen uptake at pH 6.2 in the presence of these acids was greater than for C₈ acid (Fig. 22). The corresponding methyl ketones were formed in somewhat greater amounts than in experiments with mycelium. Maximum ketone formation for the C₁₀ and C₁₂ acids between pH 6.0 and 6.5 was consistent with the finding of Frank, Platzeck & Eichhorn (1961) that β -keto lauryl decarboxylase activity was optimum at pH 6.0. The oxygen uptake by spores at pH 6.2 using the C₄ to C₁₄ fatty acids as substrates followed the same pattern as that in Fig. 9, slowly increasing after the first hour and declining after about 6 hours.

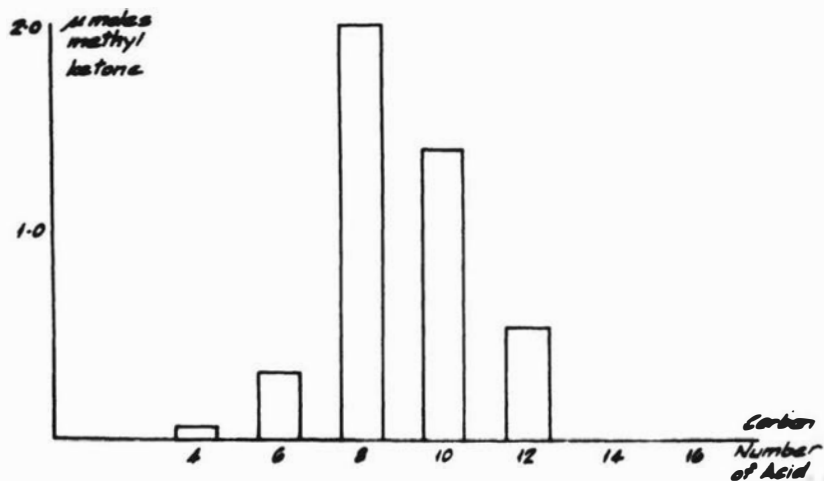
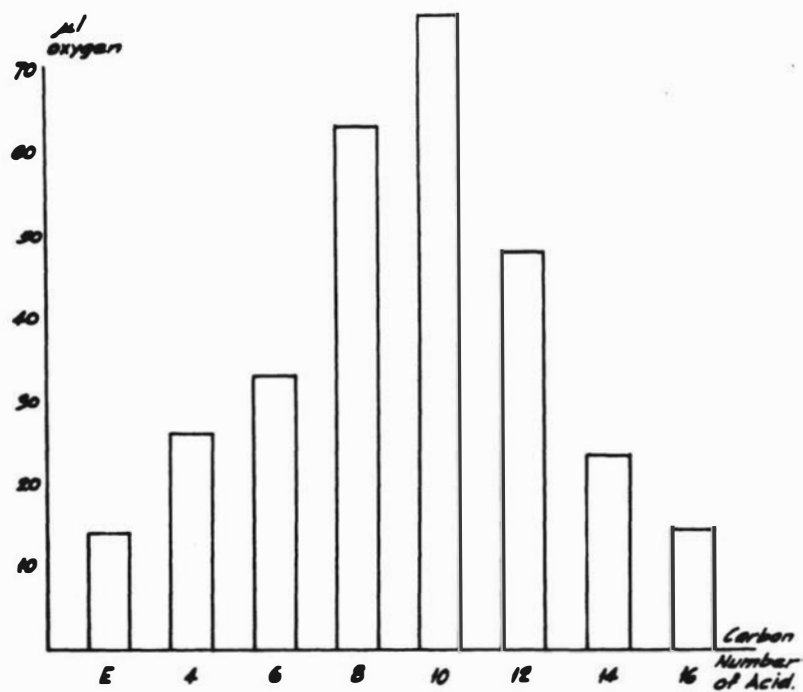


Fig.22.

Relationship between chain length of "even-numbered" fatty acids and (a) oxygen uptake (b) formation of corresponding methyl ketones, by spores at pH 6.2. Each flask contained about 1×10^7 spores, 3 μ moles of acid, 50 μ moles phosphate buffer and distilled water to 3 ml.

OXIDATION OF OCTANOIC ACID AND ITS METABOLIC PRODUCTS

Since a higher percentage of octanoic acid than the other "even-numbered" acids was oxidised to the corresponding methyl ketone at pH 5.2 and above, its metabolism was investigated in greater detail.

Oxidation of 1-C¹⁴ and 2-C¹⁴ octanoic acids by spores

The radioactivity of the 2-heptanone formed during the oxidation of labelled octanoic acids was measured, after isolation from the medium, as the 2,4-DNP hydrazone. Radioactive carbon dioxide was absorbed in the inner cells of the Warburg cells with Whatman DE 20 paper impregnated with 0.05 ml 20% KOH. The counts were obtained as detailed in the Methods section of Part II.

The question of making a correction for any fatty acid vapour that might be absorbed on the alkali impregnated paper had to be considered. At acid pH's fatty acids will exist as undissociated molecules both in solution and as vapour in the flask. The acid will however be absorbed fairly rapidly by the spores and will not then be available to volatilise from the solution. A fatty acid "blank" (i.e. a Warburg flask containing no mycelium but merely radioactive fatty acid in the phosphate medium) subtracted from the readings possibly introduces as great an error as if no correction were made. In practice "blanks" were recorded but not usually subtracted from sample counts.

Duplicate flasks containing initially 1.5 μ moles and 3 μ moles 2-C¹⁴ octanoic acid were taken off after 6 hours and 20 hours. Blank values obtained with flasks containing acid in buffer but no spores are included in Table 25 but have not been subtracted from the sample counts. The rate of appearance of ¹⁴C in respiratory CO₂ was very slow, less than 1% of the original activity of the acid (1.5 μ moles of 2-C¹⁴ octanoic acid had count of 44,750 c.p.m.) appearing as CO₂ after 6 hours incubation with spores.

2-heptanone from 1-C¹⁴ octanoic was not radioactive, showing that the carbon atom from the carboxyl group was lost during the decarboxylation. 2-heptanone from 2-C¹⁴ octanoic acid was radioactive, the specific activity of the 2,4-DNP hydrazone being very similar to that of 2-C¹⁴ octanoic acid itself. Both these findings afford strong evidence for a β -oxidation mechanism.

Oxidation of 2-C¹⁴ octanoic acid by spores in the presence of ammonium ion

The low rate of appearance of ¹⁴CO₂ prompted the question whether the enzymes required for metabolism of the acid to CO₂ were constitutive or adaptive. If the latter were the case it seemed possible that enzyme formation could be hastened by adding ammonium ion to the system. However, the presence of ammonium ion actually slightly decreased oxygen uptake both after 6 and after 20 hours (Table 26). This was unusual since aerobic oxidation of exogenous substrate is generally markedly increased if a source of nitrogen is provided

Table 25.

The oxidation of 2-C¹⁴ octanoic acid[†] by spores

Conc ⁿ acid ⁻ (μmoles)	Time (hrs)	Oxygen * uptake (μl)	2-heptanone (μmoles)	Counts per minute		
				2-heptanone	CO ₂	Acid Blank
1.5	6	40	0.48	13610	380	195
"	6	43	0.41	11445	345	165
"	20	48	0.80	26110	935	600
"	20	58	0.95	35540	760	505
3	6	56	0.95	28280	540	220
"	6	60	0.90	27145	565	210
"	20	146	2.10	71760	1685	730
"	20	140	2.01	66140	1830	710

(Each Warburg flask contained 1×10^7 spores, 12 days old, 50 μmoles phosphate buffer, pH 6.0.

* Endogenous respiration subtracted

† 1.5 μmoles 2-C¹⁴ octanoic acid had activity of 44,750 c.p.m.).

Table 26. Oxidation of 2-C¹⁴ octanoic acid by spores
in the presence of ammonium ion

Time (Hrs)	Conc ⁿ acid (μmoles)	Conc ⁿ NH ₄ ⁺ (μmoles)	Oxygen [*] uptake (μl)	2-heptanone (μmoles)	¹⁴ C ⁺ CO ₂ C.p.m.
6	1.5	-	8	0.17	240
	"	-	11	0.15	185
	"	40	3	0.14	190
	"	40	7	0.12	200
	3	-	11	0.61	330
	"	-	11	0.54	450
	"	40	11	0.22	350
	"	40	6	0.19	520
20	1.5	-	44	0.83	345
	"	-	55	0.84	290
	"	40	36	0.70	355
	"	40	39	0.68	380
	3	-	121	1.95	1040
	"	-	131	1.82	1120
	"	40	123	1.45	6545
	"	40	102	1.30	3255

(Each Warburg flask contained 1×10^7 spores, 8 days old, 50 μmoles phosphate buffer pH 6.1. 1.5 μmoles 2-C¹⁴ octanoic acid had activity of 44,750 c.p.m.

* Endogenous respiration subtracted.

† Acid blanks subtracted).

(Cochrane, 1958). The yield of methyl ketone in flasks containing ammonium ion was also decreased. In the oxidation of 1.5 μ moles 2-C¹⁴ octanoic acid, flasks without ammonium ion contained approximately 20% more 2-heptanone after both 6 hours and after 20 hours. The absence or presence of ammonium ion made no difference to the amount of ¹⁴CO₂ detected after 6 or 20 hours.

In the oxidation of 3 μ moles acid, flasks without ammonium ion contained almost 3 times more 2-heptanone after 6 hours and 40% more after 20 hours. There was no difference in ¹⁴CO₂ detected after 6 hours but a significant increase (3 to 5 times) after 20 hours with ammonium ion present.

Rates of formation of ¹⁴CO₂ during the oxidation of 1-C¹⁴ and 2-C¹⁴ octanoic acids by mycelium

Radioactive carbon dioxide was absorbed in the inner cells of the Warburg flasks by DE 20 paper impregnated with 0.05 ml 20% KOH. Duplicate flasks of each acid were analysed every 30 minutes and the counts averaged. A correction was made for any ¹⁴CO₂ absorbed, as a result of the decarboxylation of β -keto octanoic-1-C¹⁴ acid, by measuring the quantity of 2-heptanone formed. 1 μ mole of 2-heptanone is equivalent to 1 μ mole of ¹⁴CO₂, the specific activity of which would be that of the 1-C¹⁴ octanoic acid used. In this particular experiment however no methyl ketone was detected in the first 120 min. and only 0.05 μ moles after 150 min.

The rate of appearance of C^{14} in respiratory CO_2 was faster with mycelium than with spores. Less than 1% of the original activity of 2- C^{14} octanoic acid appeared as $^{14}CO_2$ after 6 hours incubation with spores (Table 25) whereas mycelium oxidised 7% of 2- C^{14} octanoic acid, and over 21% of 1- C^{14} octanoic acid, to $^{14}CO_2$ in 150 min. (Table 27).

Table 27. The effect of odd versus even carbon labelling in octanoic acid on the rate of appearance of C^{14} in respiratory CO_2

Time (mins)	Octanoic acid-1- C^{14}		Octanoic acid-2- C^{14}		Ratio Odd/even
	$^{14}CO_2$ found C.p.m.	% total C^{14}	$^{14}CO_2$ found C.p.m.	% total C^{14}	
30	1150	2.7	500	1.1	2.45
60	1955	4.5	1495	3.3	1.7
90	4380	10.1	2235	5.0	2.0
120	6365	14.7	2575	5.7	2.6
150	9430	21.7	3185	7.0	3.1

(Each Warburg flask contained approximately 10 mg mycelium, 48 hours old, 50 μ moles phosphate pH 6.0. 1.5 μ moles of both 1- C^{14} octanoic acid (43,500 c.p.m.) and 2- C^{14} octanoic acid (44,750 c.p.m.) were used).

Metabolism of 2-heptanone by mycelium

The rates at which three different batches of mycelium metabolised 2-heptanone appeared to depend upon the length of time for which the mycelium had been grown (Table 28). In Expt. 1 the oxygen uptake in excess of endogenous respiration by 48 hours old mycelium incubated with 0.2 and 0.4 μ moles of 2-heptanone was very small. The same concentrations of 2-heptanone in Expt. 2 were more rapidly metabolised by 63 hours old mycelium and a greater uptake of oxygen was recorded. In Expt. 3, 72 hours old mycelium almost completely metabolised 0.66 μ moles of 2-heptanone in 4 hours. Blank runs, i.e. shaking heptanone in the flasks without mycelium for the duration of the experiment, showed that no significant loss of ketone occurred through volatilisation.

An acceptable oxygen balance was only obtained in Expt. 2, an oxygen uptake of 60 μ l being recorded during the oxidation of 0.26 μ moles of heptanone when theoretically 58 μ l would be expected for complete oxidation to CO_2 and H_2O . It was thought possible that when non-quantitative oxygen balances were obtained part of the loss might be due to reduction of 2-heptanone to 2-heptanol by DPN (diphosphopyridinenucleotide) linked reductase (Franke, Platzeck & Eichhorn, 1962). The addition of malate, which was expected to regenerate the reduced coenzyme, made no appreciable difference to the rate of metabolism of the 2-heptanone (Table 28).

Table 28. The metabolism of 2-heptanone by mycelium

		Oxygen uptake (μ l)	2-heptanone (μ moles)	
			Initial	Final
<u>Experiment 1</u>	Mycelium, endogenous	107	-	-
Mycelium	" + 0.4 μ moles heptanone	128	0.4	0.25
48 hours old	" " " " + 0.2 μ moles malate	114	0.4	0.25
Period of incubation	" " " " + 0.2 " " (no KOH)	-	0.4	0.22
18 hours.	" + 0.2 " "	121	0.2	0.11
	" " " " + 0.2 " "	116	0.2	0.12
	" + 0.2 μ moles malate	113	-	-
	No mycelium + 0.4 μ moles heptanone	-	0.4	0.34
<u>Experiment 2</u>	Mycelium, endogenous	156	-	-
Mycelium	" + 0.4 μ moles heptanone	216	0.4	0.14
63 hours old	" " " " + 0.2 μ moles malate	309	0.4	0.17
Period of incubation	" " " " " " "	329	0.4	0.14
7 $\frac{1}{2}$ hours	" + 0.2 " malate	243	-	-
	" + 0.4 " heptanone (No KOH)	-	0.4	0.11
	" " " " + 0.2 μ moles malate (no KOH)	-	0.4	0.16
<u>Experiment 3</u>	Mycelium, endogenous	388	-	-
Mycelium	" + 0.33 μ moles heptanone	411	0.33	0.10
72 hours old	" + 0.66 " "	414	0.66	0.06
Period of incubation	No mycelium + 0.33 " "	-	0.33	0.28
4 hours	" " + 0.33 " "	-	0.66	0.59

(Each Warburg flask contained 3 mg fresh mycelium, 50 μ moles phosphate buffer pH 6.0).

The metabolism of 2-heptanone-1-C¹⁴ by mycelium

No significant percentage of the radioactivity of the 2-heptanone-1-C¹⁴ that was metabolised appeared as ¹⁴CO₂ after 3½ hours (Table 29). Blanks were obtained by shaking 2-heptanone-1-C¹⁴ in the absence of mycelium.

The observed oxygen uptake was greater than that theoretically necessary to account for the complete oxidation to CO₂ and H₂O of the heptanone metabolised by the mycelium. Since however no ¹⁴CO₂ was detected the increased oxygen uptake was presumably due to stimulation of endogenous respiration in the presence of 2-heptanone.

The metabolism of 2-heptanone-1-C¹⁴ by spores

Triplicate flasks of the radioactive 2-heptanone were analysed after incubation with spores for 8 hours. Approximately 40% of the radioactivity of the 2-heptanone-1-C¹⁴ that was metabolised appeared as ¹⁴CO₂ (Table 30).

In flask 1 the uptake of oxygen over endogenous respiration was approximately the amount that was needed for complete oxidation of the methyl ketone that disappeared. Since however only about 40% of the radioactivity of the 2-heptanone-1-C¹⁴ metabolised re-appeared as ¹⁴CO₂, it would seem that the other 60% was assimilated into endogenous material.

Table 29. The metabolism of 2-heptanone-1-C¹⁴
by mycelium

Warburg flask	2-heptanone-1-C ¹⁴ (μmoles)		Oxygen* uptake (μl)	¹⁴ CO ₂ C.p.m.
	Initial	Final		
1	0.34	0.15	43	70
2	"	0.18	36	65
3	0.19	0.12	49	60
4	"	0.10	53	55
5	0.34	0.30	-	60
6	0.19	0.18	-	50

(Each Warburg flask contained approximately 10 mg mycelium, 40 hours old, 50 μmoles phosphate buffer pH 6.0. Period of incubation 3.5 hours. Flasks 5 and 6 contained no mycelium. * Endogenous respiration deducted).

Table 30. The metabolism of 2-heptanone-1-C¹⁴
by spores

Warburg flask	2-heptanone-1-C ¹⁴ (μmoles)		Oxygen* uptake (μl)	¹⁴ C ¹⁴ O ₂ C.p.m.	¹⁴ C ¹⁴ O ₂ as % ketone metabolised
	Initial	Final			
1	0.25	0.17	18	1730	43
2	"	0.16	15	1735	39
3	"	0.13	15	1685	30
4	"	0.23	-	180	-
5	"	0.22	-	155	-

(Each Warburg flask contained 1×10^7 spores, 6 days old, 50 μmoles phosphate buffer pH 6.1. Period of incubation 8 hours. Flasks 4 and 5 contained no spores.

* Endogenous respiration deducted).

Formation of methyl carbinols during the oxidation of fatty acids

Since the question of whether methyl ketones were formed from, or converted to, methyl carbinols had yet to be resolved, it was thought desirable to re-investigate the problem using both mycelium and spores.

Octanoic acid (10 μ moles/ml) was incubated with spores at room temperature, samples being withdrawn every 24 hr. to determine whether the methyl ketone or the methyl carbinol accumulated preferentially. 2-heptanol was detected initially in greater concentration (maximum yield 1.83 μ moles/ml after 2 days) than 2-heptanone but decreased slowly to zero after 5 days (Table 31). At 28°, 2-heptanol was again detected initially in greater concentration (maximum yield 0.5 μ moles after 2 days), decreasing to zero after 4 days incubation.

Mycelium grown from the same batch of spores formed a small amount of 2-heptanol at room temperature (Table 31) but none was detected at 28°.

These experiments did not confirm the finding of Franke, Platzeck & Eichhorn (1962) that methyl carbinols accumulate during the oxidation of fatty acids by fungal spores at the same rate as the yield of methyl ketone decreases.

The relationship between the metabolism of 2-heptanone and 2-heptanol by mycelium

Fresh mycelium was shaken at 28° in two Warburg flasks, each containing 80 ml of phosphate buffer, pH 6.1

Table 31. Rates of formation of 2-heptanone and 2-heptanol during oxidation of octanoic acid (10 μ moles/ml) by spores and mycelium at room temperature

Incubation Period (days)	2-heptanone (μ moles/ml)	2-heptanol (μ moles/ml)	<u>2-heptanone</u> 2-heptanol
<u>Spores</u>			
1	0.31	0.63	0.51
2	1.02	1.83	0.56
3	1.7	0.43	4.0
4	2.0	0.09	23.0
5	2.0	Nil	-
<u>Mycelium*</u>			
2	Nil	Nil	-
3	0.05	Nil	-
4	0.22	0.03	7.0

(Each Warburg flask contained either 1×10^8 spores, 9 days old, or 100 mg mycelium, 4.8 hours old; 5000 μ moles phosphate buffer (pH 6.1) per 100 ml.

* Grown from same batch of spores).

and 0.55 $\mu\text{moles/ml}$ of either 2-heptanol or 2-heptanone. At intervals 10 ml of medium were withdrawn for ether extraction and analysis by gas chromatography as previously described and 1 ml was withdrawn for the determination of the concentration of 2-heptanone.

The rate of disappearance of the 2-heptanol from the medium was slow initially, 2% only of the 2-heptanol having been metabolised after 4 hours and 5% after 8 hours. However 75% had disappeared after 24 hours and 89% after 32 hours (Fig. 23). The yield of ketone obtained did not correspond exactly to the amount of carbinol that disappeared, the ketone presumably being slowly further metabolised by the mycelium.

The ketone (0.55 $\mu\text{moles/ml}$ initially) was slowly metabolised, the concentration falling by 25% to 0.41 $\mu\text{moles/ml}$ after 24 hours and by 44% to 0.31 $\mu\text{moles/ml}$ after 32 hours (Fig. 23). However, the concentration of 2-heptanol formed (5% of the initial 2-heptanone) did not increase to any significant extent between the first analysis (4 hours) and the last (32 hours).

In a second experiment a lower concentration of 2-heptanone (0.14 $\mu\text{moles/ml}$) was used. The rate of disappearance of ketone (35% in 2 days) and formation of 2-heptanol (less than 5%) was however almost identical to the first experiment.

In view of the poor yield of 2-heptanol obtained above, its formation was confirmed using 2-heptanone-1- C^{14} as substrate. 25 μmoles of 2-heptanone-1- C^{14} (750,000 c.p.m.) in 100 ml of buffer pH 6.1 were shaken with mycelium for 24 hours at 28°. The 2-heptanol-1- C^{14} was extracted as the 3,5 dinitrobenzoate and chromatographed as described in the Methods section. The derivative was detected under U.V. light, dried,

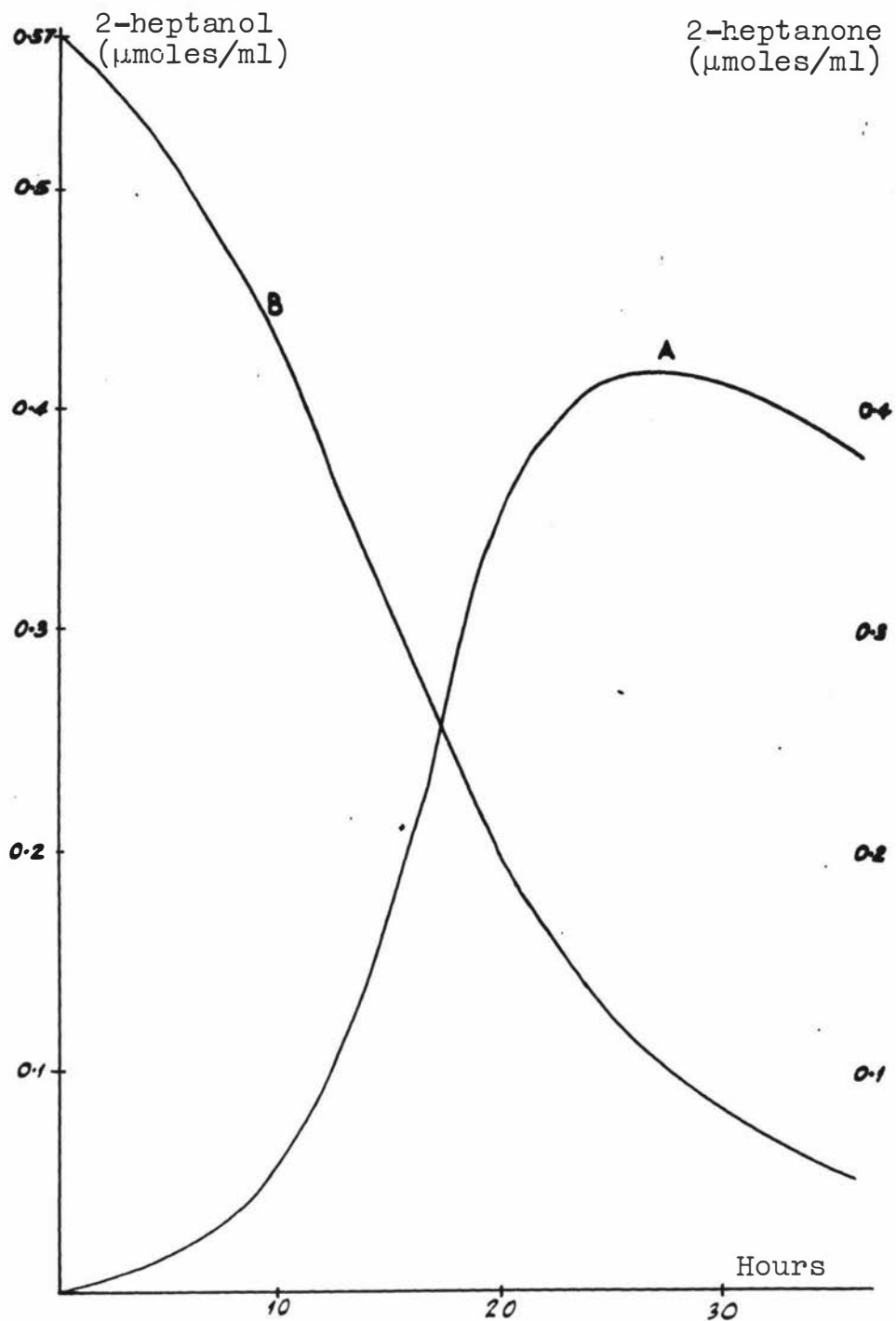


Fig.23.

The oxidation of 2-heptanol by mycelium at pH 6.1 (50 μmoles phosphate buffer/ml). Curve A shows the increase in 2-heptanone and Curve B the decrease in 2-heptanol with time.

cut out and counted directly. 1400 c.p.m. above background were obtained, which was approximately 0.2% of the original radioactivity. Although this low recovery could be partly attributed to the low yield of 3,5 dinitrobenzoate of 2-heptanol and quenching of the derivative in the scintillation fluid, it confirmed that the reduction of ketone to carbinol by mycelium occurred to a limited extent only.

Oxidation of triglycerides to methyl carbinols by spores

In contrast to the oxidation of fatty acids by spores, the oxidation of triglycerides led to a marked accumulation of both methyl carbinols and methyl ketones. For example flask C (Fig. 29) contained approximately 10 μ moles 2-heptanone and 2 μ moles 2-heptanol after 12 day old spores had been incubated with trioctanoïn and ammonium sulphate for 38 days. Flask D, in which there was no source of nitrogen and only traces of mycelium were detected, contained 11 μ moles 2-heptanone and 3.5 μ moles 2-heptanol.

Younger spores (5 days old) incubated at 30° under identical conditions to the above experiment did not however give such large quantities of carbinol. One flask contained, after 40 days incubation, 4.5 μ moles 2-heptanone and 0.25 μ moles 2-heptanol.

INHIBITION OF RESPIRATION OF MYCELIUM BY FATTY ACIDS

In the present investigation it has been shown that the ability of fatty acids to inhibit mycelial respiration depended upon

- (1) the chain length of the acid.
- (2) the concentration of acid.
- (3) the pH at which the activity was measured.
- (4) whether fresh or pre-starved mycelium was used.

There appeared to be no simple explanation available as to why the C₈ to C₁₂ acids should be more toxic than other acids. A number of supplementary experiments were therefore carried out to determine the mechanism of toxicity, particularly with reference to a possible relationship existing between toxicity and the fact that only one methyl ketone was formed in the oxidation of a fatty acid.

The combined effect of adding toxic and non-toxic acids on methyl ketone formation by mycelium

When toxic and non-toxic (e.g. C₁₁ and C₅) acids were added simultaneously, endogenous respiration was inhibited. However both corresponding methyl ketones were formed showing that the acids were oxidised as far as the β -keto acids (Table 34).

When a non-toxic acid, e.g., C₅, was first shaken with the mycelium for 30 minutes and oxidation was

occurring normally, the addition of a toxic acid such as C_{11} suppressed oxygen uptake by the mycelium markedly. The toxic acid was not immediately effective in inhibiting oxygen uptake, C_{11} acid taking 30 minutes and the less toxic C_{10} acid 45 minutes to reduce the oxygen uptake to a minimum (Fig. 24). The addition of the toxic acid however in most cases markedly increased the formation of C_4 methyl ketone from C_5 acid (Table 34), the more toxic acid (C_{11}) being more effective than the less toxic (C_{10}).

Effect of concentration of toxic acid added

The preliminary experiments had shown that although the oxygen uptake by mycelium in the presence of a non-toxic acid could be inhibited by the addition of a toxic acid, methyl ketone formation from the non-toxic acid was actually enhanced in most cases. It was of interest therefore to determine the relationship between methyl ketone formation from the non-toxic acid and the concentration of toxic acid added.

Pre-incubation with non-toxic acid (C_8) followed by toxic acid (C_{10})

2 μ moles of C_8 acid were shaken with mycelium in Warburg flasks for 30 min, varying concentrations of C_{10} acid then being added. At the same time, identical concentrations of C_{10} acid were added to fresh mycelium

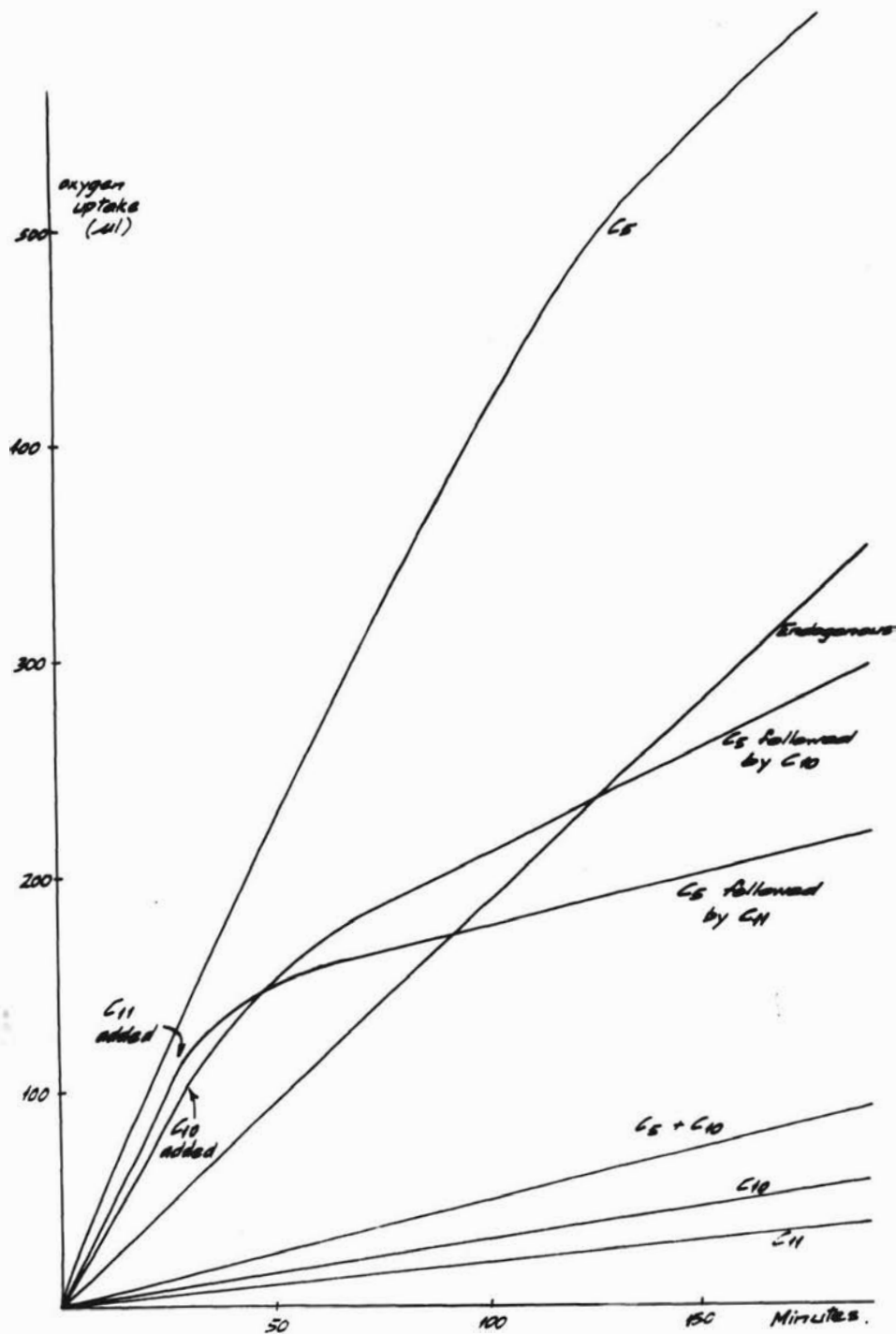


Fig.24.

The inhibition of oxygen uptake by mycelium in the presence of a non-toxic acid (C_5) by the addition of a toxic acid (C_{10} or C_{11}). Each flask contained about 10 mg mycelium, 50 μ moles of phosphate buffer, pH 5.2, 3 μ moles of each acid and distilled water to 3 ml.

Table 34. The combined effect of adding toxic and non-toxic acids on methyl ketone formation by mycelium

		Methyl ketone (μ moles)		
		C ₄	C ₉	C ₁₀
<u>Experiment 1</u>	Mycelium, 60 hrs. old			
	C ₅ only	0.19	-	-
	C ₁₀ "	-	0.40	-
	C ₁₁ "	-	-	0.16
	C ₅ & C ₁₀ together	0.39	0.37	-
	C ₅ followed by C ₁₀	0.39	0.35	-
	C ₅ " " C ₁₁	0.90	-	0.20
<u>Experiment 2</u>	Mycelium, 48 hrs. old			
	C ₅ only	0.26	-	-
	C ₁₁ "	-	-	0.30
	C ₅ followed by C ₁₁	0.90	-	0.25
	C ₁₁ " " C ₅	0.18	-	0.20
	C ₅ & C ₁₁ together	0.15	-	0.15

(Each Warburg flask contained 10 mg mycelium, 50 μ moles phosphate buffer pH 5.2. 3 μ moles of each acid were used, the second of any pair of acids being added 30 min. after the first and incubation continued for a further 160 min).

Table 35. The effect of C₁₀ acid on the ability of mycelium to oxidise C₈ acid to 2-heptanone

	2-heptanone (μ moles)	2-nonanone (μ moles)
Mycelium, control	Nil	Nil
" + 2 μ moles C ₈	0.05	Nil
" + " followed by 2 μ moles C ₁₀	0.46	0.08
" + " " " 4 " "	0.18	0.06
" + " " " 6 " "	0.05	0.08
Mycelium, shaken, " " 2 " "	-	0.14
" " " " 4 " "	-	0.10
" " " " 6 " "	-	0.10
Mycelium + 2 μ moles C ₁₀ " " 2 " C ₈	0.80	0.07
" + 3 " " " 2 " "	0.92	0.09

(Each Warburg flask contained 10 mg mycelium, 48 hours old, 50 μ moles phosphate buffer pH 5.2. The second acid was added 30 minutes after the first, incubation being continued for a further 120 min.).

as controls. As the results in Table 35 show the production of 2-heptanone was markedly increased by adding 2 μ moles C_{10} acid, but the addition of higher concentrations of C_{10} acid resulted in smaller increases of 2-heptanone from octanoic acid.

Pre-incubation with toxic acid followed by non-toxic acid

Mycelium from the above experiment was used to investigate the effect of shaking for 30 minutes with a toxic (C_{10}) acid followed by a non-toxic (C_8) acid. A considerably higher yield of 2-heptanone was obtained than with the reverse order of acids (Table 35). In view of these results mycelium was pre-incubated with different concentrations of C_{10} acid for varying times to determine whether the duration of contact of the C_{10} acid with the mycelium influenced its ability to subsequently oxidise C_8 acid to 2-heptanone.

Pre-incubation of mycelium with C_{10} acid for 3 hours.

Mycelium suspended in phosphate buffer (pH 5.2) was shaken for 3 hours with two different concentrations of C_{10} acid (4 μ moles and 10 μ moles). The mycelium was collected on a Millipore Filter, washed with 100 ml of 0.05 M phosphate buffer (pH 7.0) and with 50 ml of distilled water. The mycelium was then resuspended in 3 ml of phosphate buffer (pH 5.2). 2 μ moles of C_8 acid were added to the washed mycelium and oxidation allowed to proceed for 3 hours. The mycelium pretreated with 4 μ moles C_{10} gave an almost quantitative yield of 2-heptanone. Oxygen uptake over endogenous

respiration was very slow, the observed uptake of 42 μ l being almost that theoretically needed for the formation of 1.86 μ moles of 2-heptanone from C_8 acid (Table 36, Expt. 1). Pre-incubation of the mycelium with 10 μ moles C_{10} acid however completely inhibited its ability subsequently to oxidise octanoic acid to 2-heptanone.

Two controls were obtained by shaking mycelium in phosphate buffer, washing as described above and resuspending in pH 5.2 buffer. To one control 2 μ moles C_8 acid were added, oxygen uptake being rapid and almost quantitative after 3 hours, indicating that the mycelium was unaffected by the washing procedure. The second control showed the endogenous respiration of the washed mycelium to be the same as that before washing.

To demonstrate that the washing procedure had not removed the inhibitory effect of the C_{10} acid, mycelium pre-incubated with C_{10} acid was washed and resuspended as above. The oxygen uptake by these washed cells (Table 36) remained very low.

Pre-incubation of mycelium with C_{10} acid for 40 min. and 10 min. Different batches of mycelium were pre-incubated for 40 min. and 10 min. respectively with varying concentrations of C_{10} acid. The time for which the C_{10} acid was in contact with the mycelium did not appear to be important since in all cases the pattern of subsequent oxidation of octanoic acid to 2-heptanone by the mycelium was the same (Table 36, Expts. 2 and 3).

Table 36. The effect of pre-incubating mycelium with C₁₀ acid for varying times on its ability to oxidise C₈ acid to 2-heptanone

				2-heptanone (μmoles)	Oxygen uptake (μl)
<u>Experiment 1.</u> Pre-incubation with C ₁₀ acid for 3 hours					
Mycelium		washed,*	control	Nil	259
"	+ 4 μmoles C ₁₀	"	"	Nil	15
"	+10 " "	"	"	Nil	2
Mycelium control,		"	+ 2 μmoles C ₈ acid	0.81	578
"	+ 4 μmoles C ₁₀	"	" " "	1.86	57
"	+10 " "	"	" " "	Nil	6
<u>Experiment 2.</u> Pre-incubation with C ₁₀ acid for 40 minutes					
Mycelium		washed,*	control	Nil	202
"	+ 2 μmoles C ₁₀	"	"	Nil	10
"	+ 3 " "	"	"	Nil	3
Mycelium		"	+ 2 μmoles C ₈ acid	0.80	438
"	+ 2 μmoles C ₁₀	"	" " "	1.30	75
"	3 " "	"	" " "	1.02	69
"	4 " "	"	" " "	0.24	15
"	6 " "	"	" " "	0.12	15
<u>Experiment 3.</u> Pre-incubation with C ₁₀ acid for 10 minutes					
Mycelium		washed,*	control	Nil	179
"		"	+ 2 μmoles C ₈ acid	0.27	414
"	+ 2 μmoles C ₁₀	"	" " "	1.53	68
"	4 " "	"	" " "	0.50	33
"	6 " "	"	" " "	0.21	32
"	8 " "	"	" " "	0.10	30
"	10 " "	"	" " "	0.09	36

(Each Warburg flask contained 10 mg washed* mycelium, 48 hours old, 50 μmoles phosphate buffer pH 5.2. Period of incubation 3 hours. * see text for details).

The use of 2-C¹⁴ octanoic acid to show the extent of toxicity exerted by C₁₀ acid

To investigate further the toxic action of C₁₀ acid on mycelium, experiments were carried out using 2-C¹⁴ octanoic acid. The mycelium was shaken for 1 hour with 6 μmoles of C₁₀ acid, washed with pH 7.0 buffer as outlined in the previous experiments and re-suspended in buffer (pH 5.2). 1 μmole of 2-C¹⁴ octanoic acid was then incubated with the washed mycelium for 2 hours. An acid blank, i.e. 1 μmole 2-C¹⁴ octanoic acid in 3 ml buffer (pH 5.2) without added mycelium, was also included.

Mycelium washed with buffer as above was able to oxidise octanoic acid to carbon dioxide. In 2 hours 5 to 10% of the initial radioactivity of the acid appeared as radioactive CO₂ (Table 37). Mycelium that had been pre-treated with C₁₀ acid formed no significant amounts of radioactive CO₂ when incubated with 2-C¹⁴ octanoic acid. A greater yield of 2-heptanone-1-C¹⁴ was obtained after using mycelium pre-treated with C₁₀ acid than with fresh mycelium.

Reversibility of toxicity of octanoic acid at low pH

The dependence of the toxic effect of acids upon pH has been amply demonstrated in the previous experiments. The results in Fig. 25 show that the toxic effect of C₈ on mycelium at pH 2.5 could be reversed slowly by the addition after 1 hour of sufficient KOH (30 μmoles) to bring the contents of the Warburg cell to pH 5.5, at which pH C₈ acid is normally oxidised

Table 37. The use of octanoic-2-C¹⁴ acid to demonstrate the extent of toxicity exerted by C₁₀ acid

	Oxygen uptake (μ l)	¹⁴ C ₂ O ₂ C.p.m	2-heptanone -1-C ¹⁴ (μ moles)
Mycelium, washed. Control	344	-	-
" " "	312	-	-
" " + 1 μ mole octanoic-2-C ¹⁴ acid	388	2400	0.05
" " " " "	445	2120	0.12
" + 6 μ moles C ₁₀ acid, washed. Control	40	-	-
" " " " + 1 μ mole octanoic-2-C ¹⁴ acid	74	230	0.31
" " " " " " " "	64	225	0.28
No mycelium + 1 μ mole octanoic-2-C ¹⁴ acid (blank)	-	215	-

(Each Warburg flask contained 10 mg washed mycelium, 60 hours old, 50 μ moles phosphate buffer pH 5.2. Period of incubation 2 hours).

Table 38. The reversibility of the toxicity of octanoic acid at pH 2.6 towards mycelium by addition of alkali

Octanoic acid μmoles	pH	2-heptanone μmoles
4	2.6	0.12
6	"	0.05
8	"	0.03
4	5.5	2.61
6	"	2.65
8	"	2.38
4	2.6 → 5.5*	2.33
6	" "	1.80
8	" "	0.62

(Each Warburg flask contained 10 mg mycelium, 48 hours old, 50 μmoles phosphate buffer. Incubation period 6 hours.

* 30 μmoles KOH added after 1 hour to raise pH from 2.6 to 5.5).

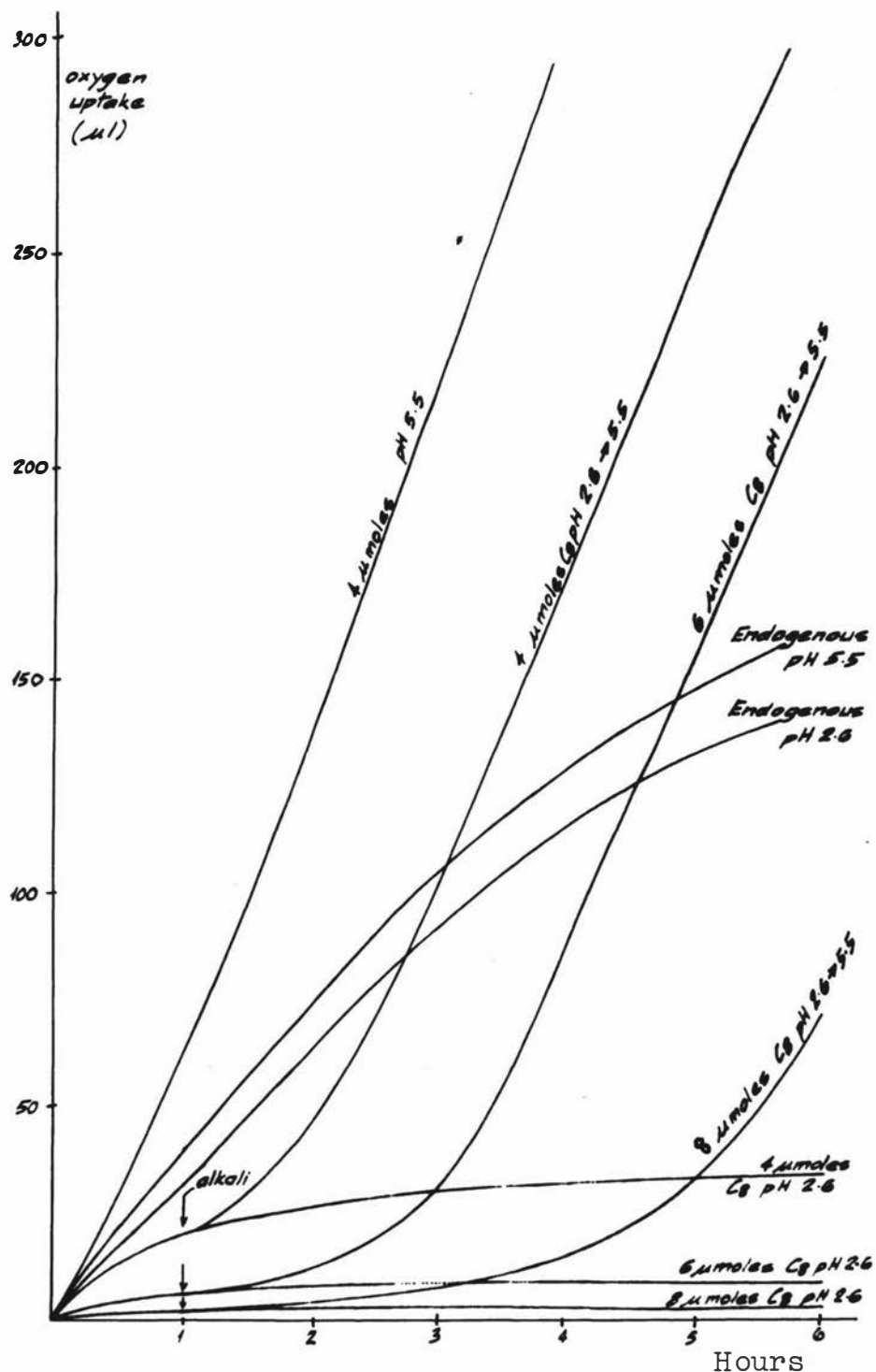


Fig.25. The reversibility of the toxic effect of octanoic acid (4, 6 and 8 μ moles) towards mycelium at pH 2.6 by raising the pH to 5.5. Each flask contained about 10 mg mycelium (dry weight), 50 μ moles phosphate buffer and distilled water to 3 ml. 30 μ moles KOH added after 1 hour to raise pH to 5.5.

readily. The oxygen uptake and yield of 2-heptanone slowly increased after the addition of alkali. The yield of 2-heptanone obtained from 4 μ moles octanoic acid at pH 5.5 was not significantly higher than that when the acid was first added at pH 2.6 and the pH then raised after 1 hour to 5.5 (Table 38).

The time taken to overcome the toxicity at pH 2.6 depended upon the concentration of toxic acid added initially (Fig. 25). This was also shown by the relatively low yield of 2-heptanone obtained when the pH of the flask containing 8 μ moles of octanoic acid was raised from 2.6 to 5.5 (Table 38).

Effect of $-\text{CO}-\text{CH}_2-\text{CO}$ grouping on oxidation of fatty acids

A working hypothesis considered was that the β -keto acids themselves were toxic to the oxidising enzymes. Both $-\text{NH}_2$ and $-\text{SH}$ groups might be expected to add readily on to the β -keto group in the $-\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$ grouping since the β -keto group will be activated by the proximity of the $-\text{COOH}$ group. The only readily available compound containing a $-\text{CO}-\text{CH}_2-\text{CO}$ grouping was ethyl acetoacetate. This was rapidly oxidised by mycelium at pH 5.8 and its presence was also unable to prevent the oxidation of octanoic acid to any significant extent. However the presence of acetoacetate partly inhibited the oxidation of C_{10} acid (Table 39); the oxygen uptake was approximately halved and the formation of methyl ketone decreased by one third.

Table 39. The effect of ethyl acetoacetate (EtAcAc) and glutathione (GSH) on the oxidation of fatty acids by mycelium

	Oxygen uptake (μ l)	Methyl ketone (μ moles)
Mycelium, Endogenous	54	-
" + 3 μ moles C ₁₀ acid	351	0.31
" + " " + 100 μ moles EtAcAc	192	0.19
" + " " + 100 " GSH	25	0.26
" + 100 μ moles EtAcAc	450	-
" + 100 " GSH	93	-
" + 2 μ moles C ₈ acid	295	1.07
" " " + 25 μ moles GSH	265	0.56
" " " 50 " "	216	0.40
" " " 100 " "	200	0.38
" " " 100 " EtAcAc	300	0.94

(Each Warburg flask contained 10 mg mycelium, 48 hours old, 50 μ moles phosphate buffer, pH 5.8. Incubation period 4 hours).

Effect of glutathione

An attempt was also made to determine if the toxicity of fatty acids towards mycelium was a consequence of the deactivation of the thiol groups of the oxidising enzymes. The presence of glutathione was expected to protect such enzymes. An unexpected result was that the glutathione, although somewhat stimulatory to the control, both suppressed the oxygen uptake during the oxidation of C₈ and C₁₀ acids and lowered the yield of methyl ketone (Table 39).

OXIDATION OF TRIGLYCERIDES

Oxidation of triglycerides by mycelium

Mycelium oxidised triglycerides slowly, after an initial lag period of about 1 hour (Fig. 26). This lag period was possibly caused by the necessity for the cells to synthesise lipases, after which accelerated oxidation took place. High lipase activity might not be expected in mycelium grown with the glucose-ammonium nitrate medium since many workers (e.g. Thibodeau & Macy, 1938; Peters & Nelson, 1948) have shown glucose to inhibit lipase formation. This was confirmed by growing the mycelium with casamino acids as the sole source of carbon. In this case oxidation was rapid and commenced immediately (Fig. 26). Methyl ketones were not detected as oxidation products of fatty acids incubated for 5 hr. with mycelium grown on either medium.

Effect of shaking on lipolytic activity. Since aeration has been found to deactivate other lipases (Nelson, 1952) it was possible that shaking the Warburg flasks was influencing lipase activity. However, no difference in activity was observed between flasks shaken and those that were not.

Oxidation of triglycerides by spores

Small quantities of free fatty acids (1 μ mole/ml) were oxidised by spores to yield up to 60% of the corresponding methyl ketone after 6 hours but only small amounts of methyl ketone were obtained from equivalent quantities

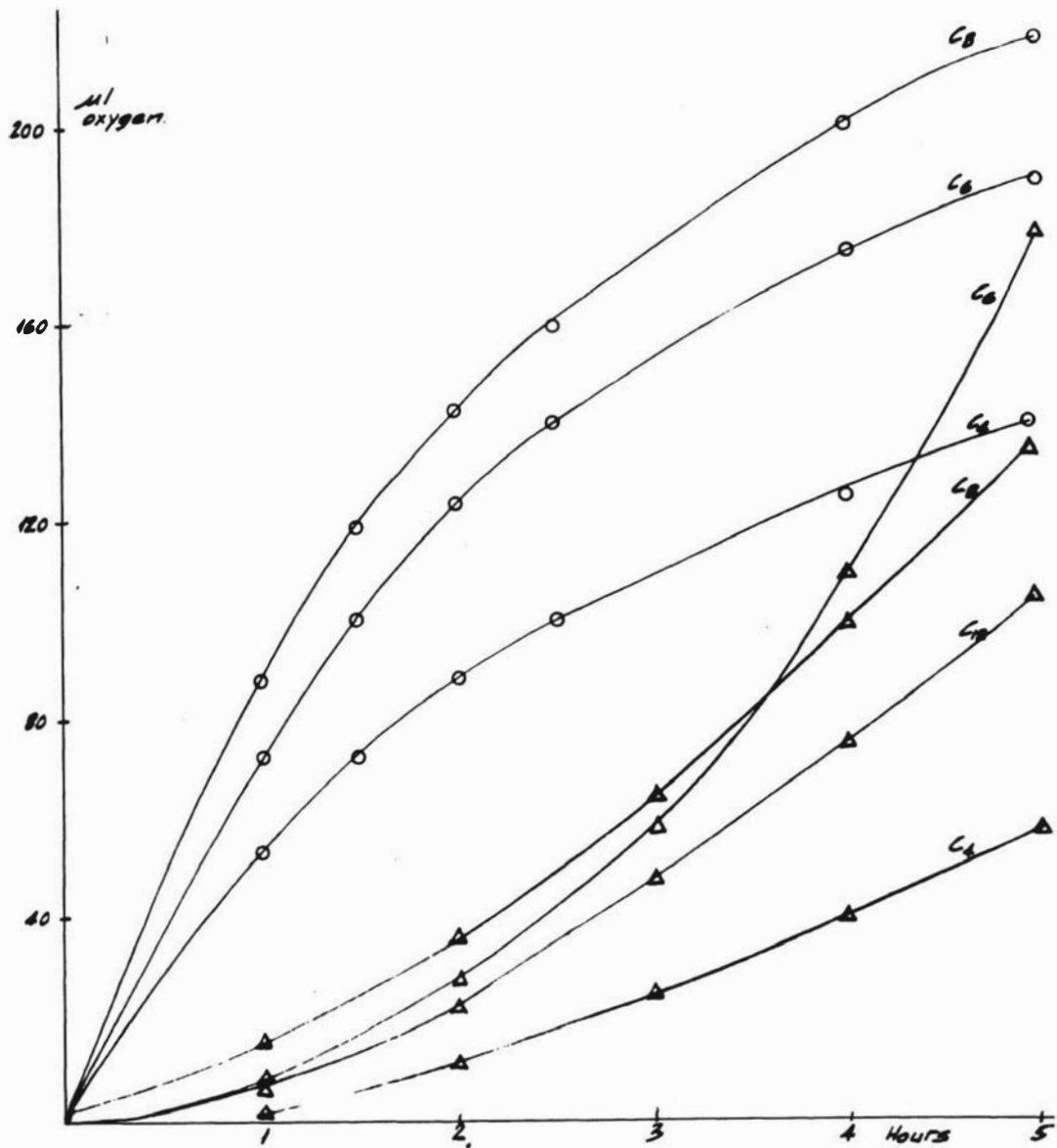


Fig.26. Comparison of the rates of oxidation of triglycerides by mycelium grown on casamino acids (o - o) and on glucose - ammonium nitrate (Δ - Δ). Each flask contained about 10 mg mycelium, 1 μ mole of triglyceride and 50 μ moles phosphate buffer, pH 5.2. Endogenous respiration deducted.

of triglycerides (Fig. 27) in the same period. Similarly the oxygen uptake was much higher for the free acids than for the triglycerides, trilaurin not being oxidised at all. These preliminary experiments indicated that the lipolytic activity of the spores was comparatively low. Over a period of weeks however spores were found to oxidise slowly large concentrations of triglyceride to methyl ketone whereas equivalent concentrations of free fatty acid were not oxidised. 0.2 ml trioctanoin, i.e. 44 μ moles "available" octanoic acid per ml, was oxidised to 10.1 μ moles 2-heptanone after 9 days and 0.3 ml trioctanoin to 6.6 μ moles after 14 days (Table 40). Above these concentrations however the formation of 2-heptanone was markedly decreased. Thus 0.14 ml trioctanoin yielded only 0.6 μ moles 2-heptanone after 14 days.

The effect of age of spores on methyl ketone formation.

No correlation was observed between the age of spores and methyl ketone formation from triglycerides (Fig. 28), a result similar to that obtained in the oxidation of free fatty acids by spores.

Induction of lipolytic activity in germinating spores

The preliminary step in the oxidation of triglycerides to methyl ketones is presumably hydrolysis of the triglyceride to free acid. Spores grown on Czapek-Dox agar gave considerably higher yields of 2-heptanone from trioctanoin than either spores grown on potato dextrose agar (Fig. 28) or spores grown on malt agar.

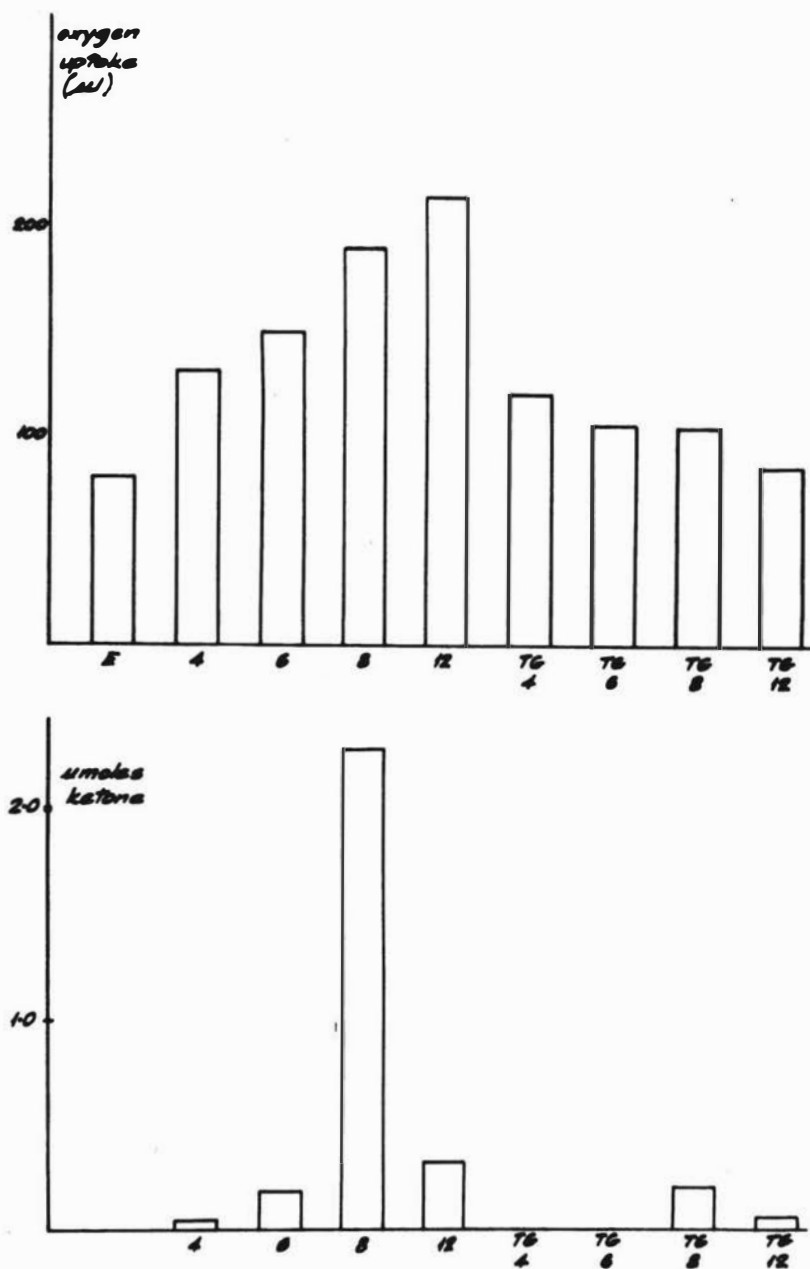


Fig.27. A comparison of the oxygen uptake and methyl ketone formation by 4 day old spores in the presence of (a) fatty acids and (b) triglycerides. Each flask contained either 3 µmoles of fatty acid or 1 µmole of the corresponding triglyceride, about 1×10^7 spores, 50 µmoles phosphate buffer, pH 6.2 and distilled water to 3 ml. Incubation period 6 hours. E = endogenous respiration. TG = triglyceride.

Table 40. The effect of concentration of trioctanoin on the formation of methyl ketones by 15 day old spores at 30°

Concentration trioctanoin (ml)	"Available" octanoic acid (μ moles/ml)	Yield of methyl ketone (μ moles/ml)					
		Days of incubation					
		2	5	9	14	19	36
0.1	22	1.6	2.6	3.1	3.6	3.2	3.1
0.2	44	3.1	4.6	10.1	6.3	6.0	6.0
0.3	66	1.3	4.2	4.8	6.6	5.9	4.1
0.4	88	0.2	0.3	0.4	0.6	0.5	0.7
0.5	110	0.05	0.05	0.08	0.08	0.09	0.07

(Each flask contained 25 ml phosphate buffer pH 6.6; 7 ml spore suspension (1×10^6 spores per ml)).

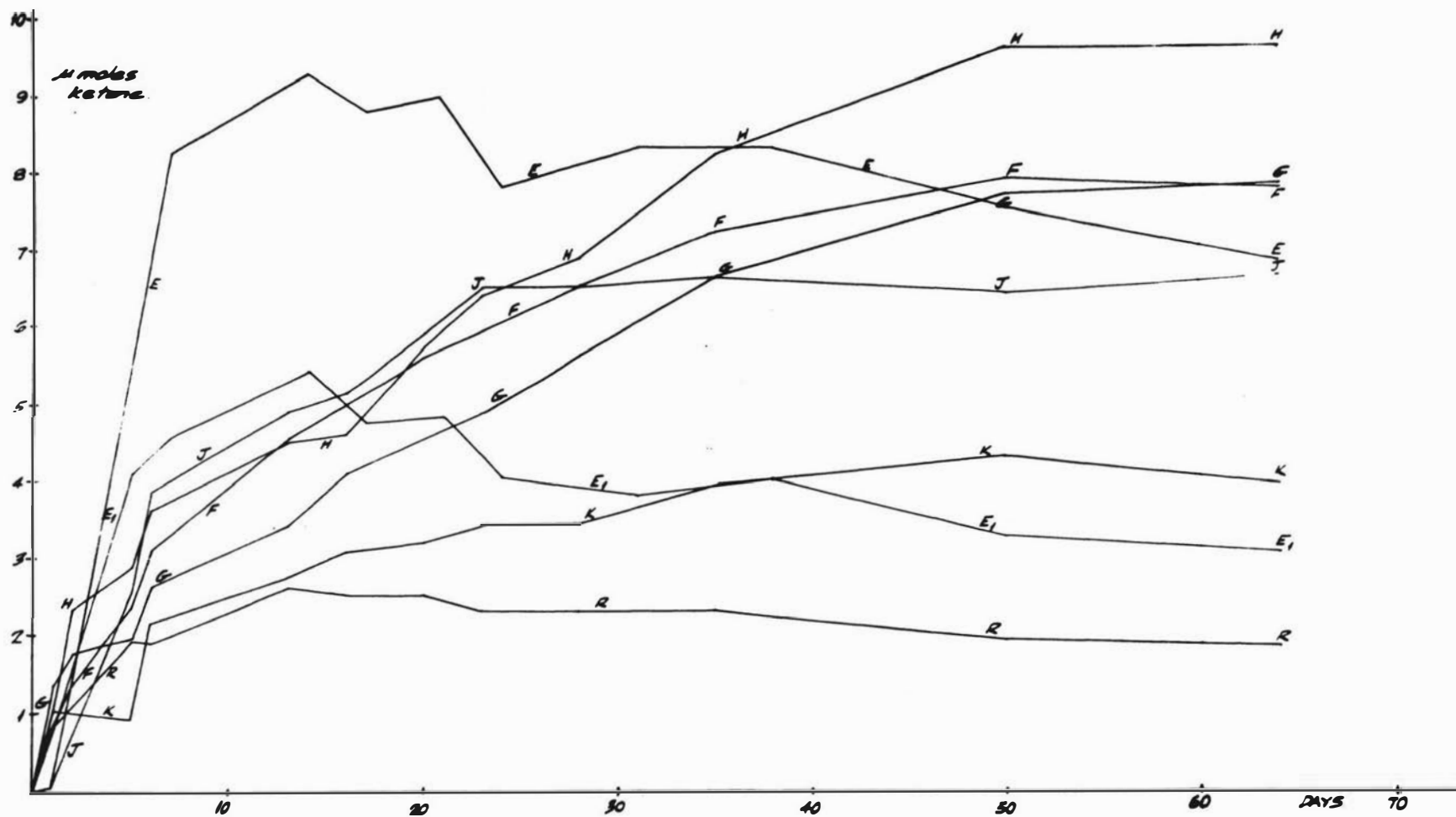


Fig. 28. Comparison of 2-heptanone formation from trioctanoin by spores grown on (a) Czapek-Dox agar (E, F, G, H) (b) Potato-dextrose agar (E₁, J, K, R). Spores used in flasks E, E₁, F and R were 6 days old; those in G and J were 13 days old and in H and K were 20 days old. Flasks E and E₁ contained ammonium sulphate (50 μmoles/ml).

These three media contain different concentrations of sugar (and, in the case of malt agar, maltose instead of glucose) which may have affected the relative lipolytic behaviour of the spores. Glucose is known to inhibit lipase formation in mycelium (e.g. Thibodeau & Macy, 1938) and could reasonably be expected to inhibit their formation in spores also. Attempts were made to increase the lipolytic activity of spores by growing them on glucose-free media, whose carbon source was casamino acids, peptone, neopeptone or tryptone, but the mycelium did not sporulate.

Effect of nitrogen source on lipolytic activity. Since bacterial lipase production is markedly affected by the source of nitrogen in the growth medium (Cutchins, Doetsch & Pelczar, 1952), it was thought that lipases might be induced by adding various sources of nitrogen to the incubation medium. The ability of spores to oxidise triglycerides to methyl ketones in a medium containing no source of nitrogen was compared with their ability to do so in media containing various sources of nitrogen. Although spores can germinate in absence of nitrogen there is little or no post germinative development (Levinson & Hyatt, 1963) and thus these experiments allowed a comparison to be made between germinative and post-germinative influence on methyl ketone formation.

(a) Casamino acids (3g/500 ml). The oxidation of triglycerides to methyl ketones by spores was much less marked in the presence of casamino acids than in experiments with ammonium ion reported below. The presence of casamino acids resulted in extensive mycelial

growth which appeared to be correlated with the low yields of methyl ketones obtained.

(b) Ammonium sulphate (1g/500 ml). At pH 6.3 spores oxidised samples of trioctanoin (22 μ moles/ml) more rapidly in the presence of ammonium ion than those in its absence (Fig. 29).

Formation of methyl ketone in the latter was however still increasing after 36 days whereas those with ammonium ion had reached a maximum after 10-20 days. All flasks containing ammonium ion formed some mycelium but further metabolism of the 2-heptanone was very slow.

A similar series of experiments was carried out at the same time with the strain of P. roqueforti isolated from N.Z. Blue cheese. Although the maximum yield of ketone (12 μ moles/ml) was not as high as obtained with strain 6989, the pattern of ketone formation was similar.

Although in general the presence of ammonium ion initially accelerated the formation of methyl ketones from triglycerides it was found that far more consistent results were obtained, if rather more slowly, by spores in the absence of a source of nitrogen (see, for example, Fig. 28). A feature of the experiments concerned with the metabolism of triglycerides in the presence of ammonium ion was the difficulty of exactly reproducing results in different trials employing comparable cultures.

Effect of calcium, magnesium and chloride ions. Addition of 0.02% (w/v) calcium chloride, frequently added as an activator in lipase systems (Bier, 1955), sometimes greatly enhanced, and at other times repressed, methyl ketone formation (Figs. 29, 30, 32). Similarly inconsistent

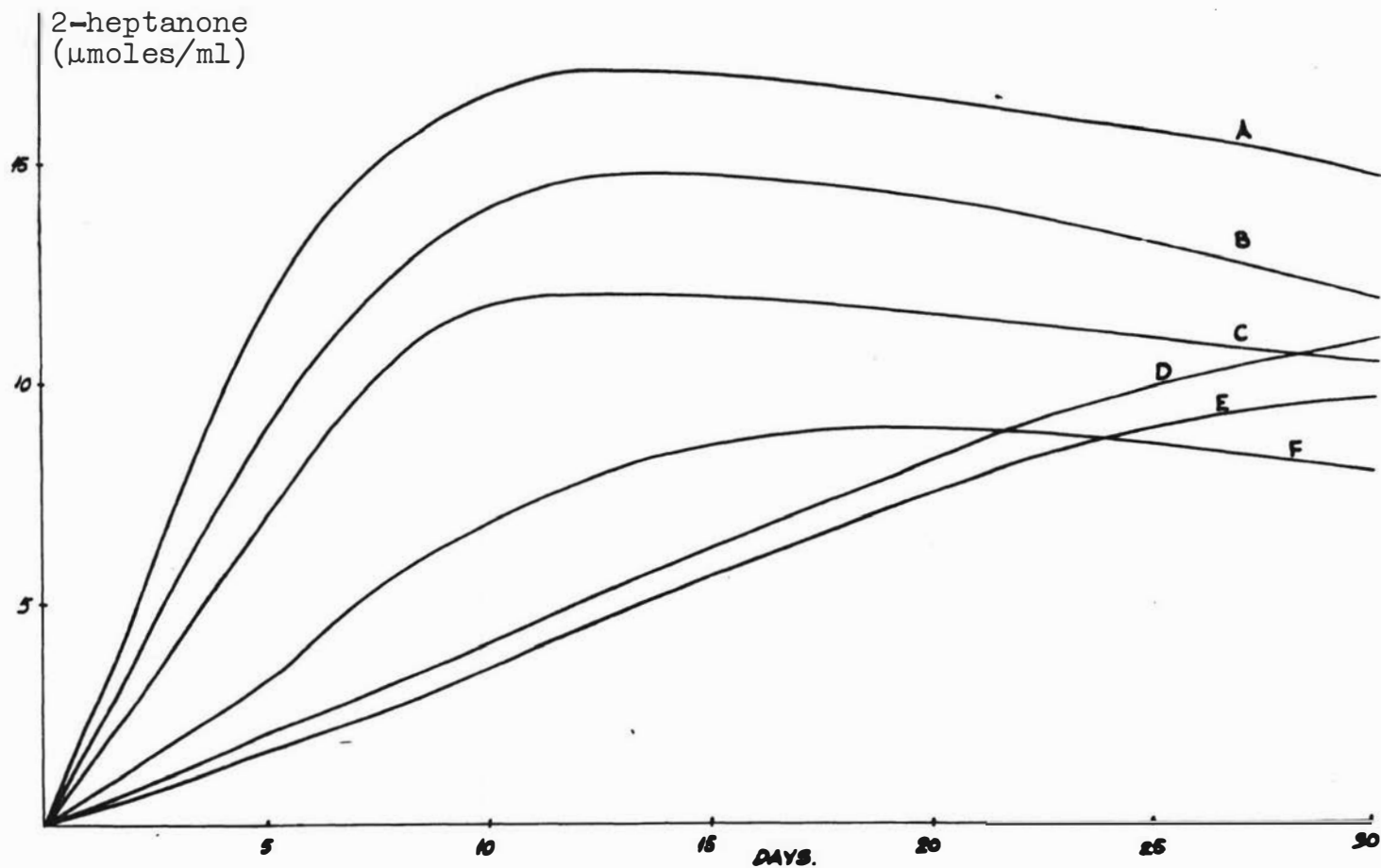


Fig.29. A comparison of the formation of 2-heptanone from trioctanoin by 12 day old spores at pH 6.3 (50 μ moles phosphate buffer/ml) in the presence or absence of ammonium sulphate (50 μ moles/ml) and 0.02% (w/v) calcium chloride. Flasks, A, B, C and F contained ammonium ion: flasks A, E, F, calcium chloride and flask D neither ammonium ion nor calcium chloride.

results were obtained when magnesium ion was added.

Bier (1955) reported halogen ions to be inhibitory but in this investigation the presence of chloride ion did not significantly alter the amount of methyl ketone formed.

Effect of emulsification of triglycerides. Many workers, e.g., Kirsch, 1935; have stated that emulsification is a pre-requisite for lipolysis of triglycerides. Trioctanoin was therefore emulsified with 0.2% solutions of gum acacia, agar or polyvinyl alcohol, prepared according to methods given by Bier (1955). Emulsification did not however enhance ketone formation (Fig. 30).

The formation of 2-undecanone from Tween 20 by spores

The solubility in water of Tween 20 (polyoxyethylene sorbitan monolaurate), which contains approximately 60% lauric acid (Archibald, 1946), was utilised to determine if the low rate of formation of 2-undecanone from trilaurin was due to the insolubility of the triglyceride. Formation of 2-undecanone from Tween 20 was linear but very slow for about 10 days and then stopped completely (Fig. 31). This experiment would appear to confirm that emulsification or solubility of the substrate triglyceride is not critical.

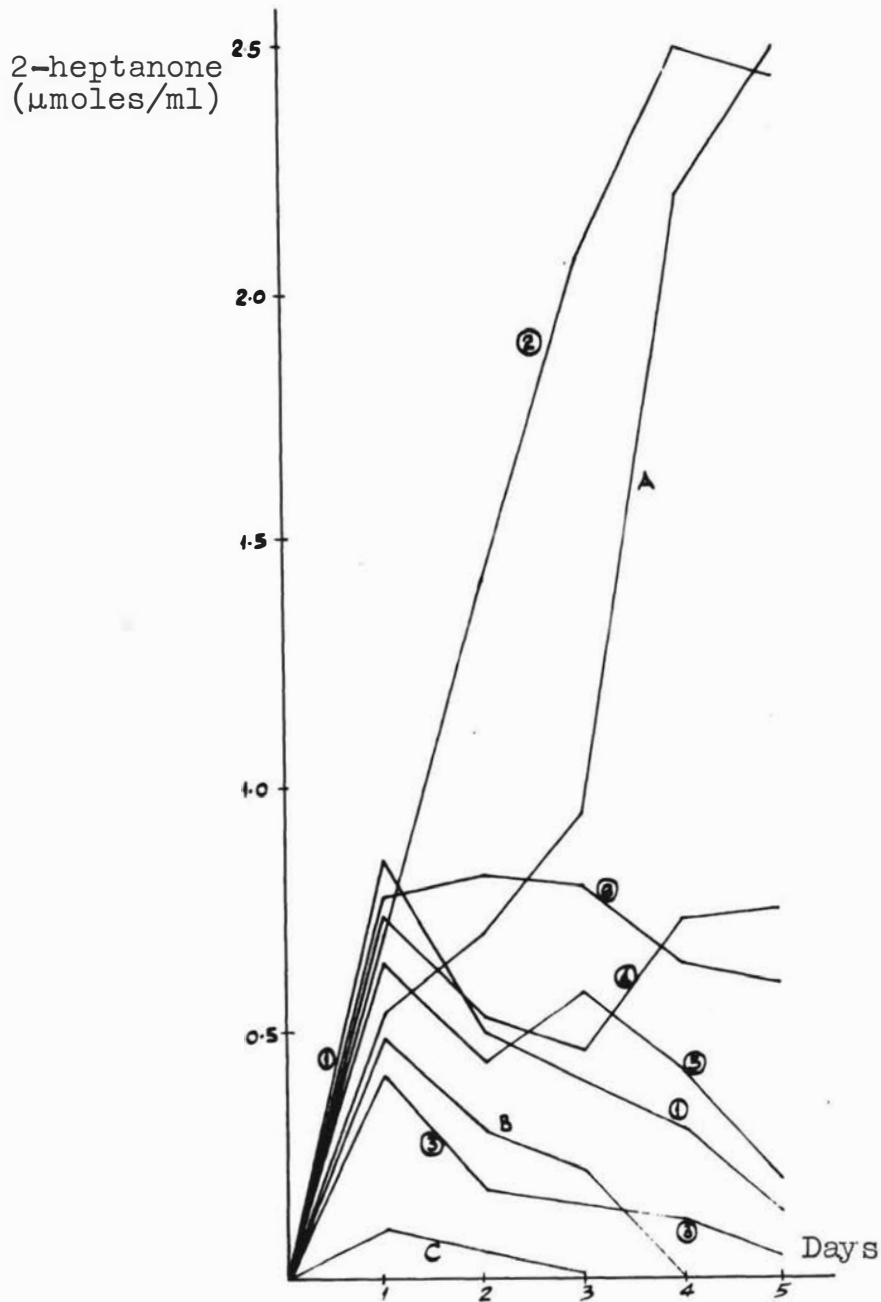


Fig.30. Effect of emulsifying 0.2% trioctanoin with gum acacia or polyvinyl alcohol on subsequent formation of 2-heptanone by spores. All flasks contained phosphate buffer pH 6.5 (50 $\mu\text{moles/ml}$) and ammonium sulphate (50 $\mu\text{moles/ml}$); flasks 2,4,6 and B contained 0.2% CaCl_2 . In flasks 3 and 4 the trioctanoin was emulsified with 0.2% gum acacia and in flasks 5 and 6 with 0.2% polyvinyl alcohol.

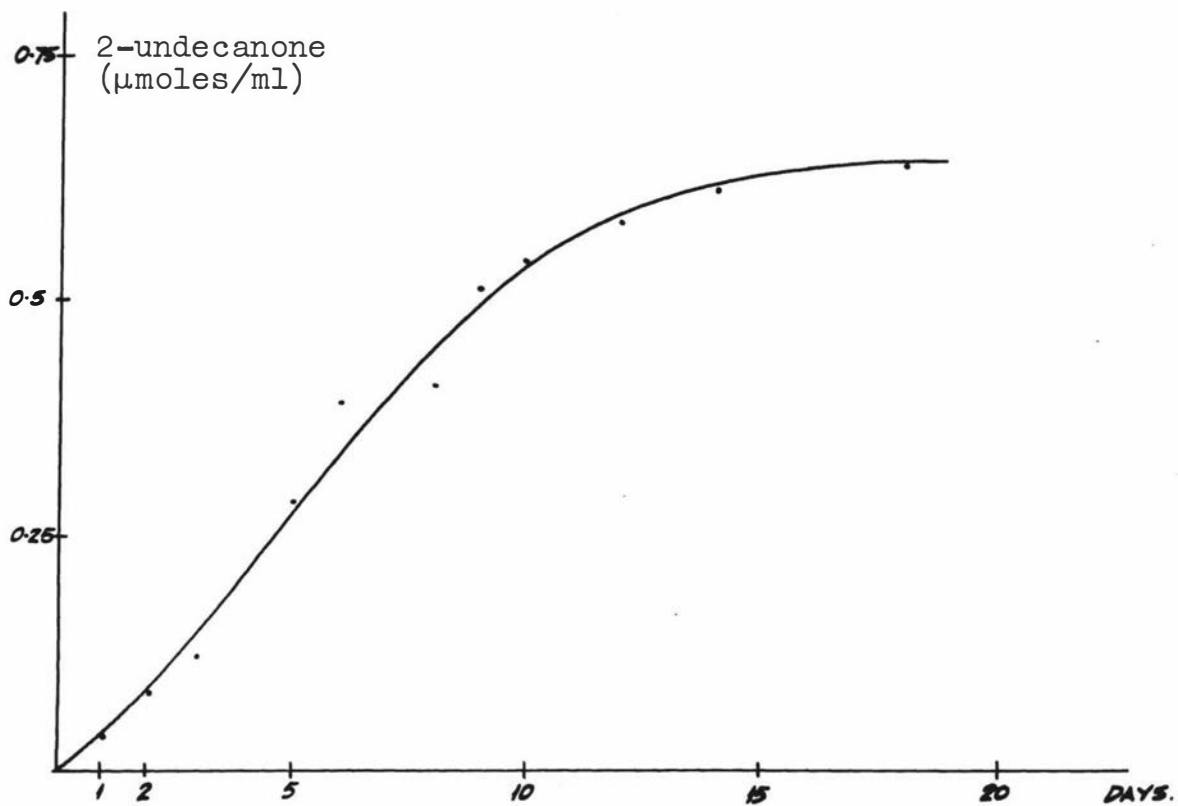


Fig.31. The formation of 2-undecanone from Tween 20 (polyoxyethylene sorbitan monolaurate) by 10 day old spores at pH 6.5 (M/10 phosphate buffer).

Effect of pH of incubation medium. Since both the initial lipolysis of the triglyceride and the further oxidation of free acid to methyl ketone are pH dependent, it was of interest to determine the effect of pH on the oxidation of triglycerides by spores in media with and without ammonium ion.

In the presence of ammonium ion the highest yields of corresponding methyl ketones formed at pH 2.5 and 5.2 were from tributyrin, and at pH 6.5 from trihexanoin, (Table 41). At pH 2.5 the ketones formed were not further metabolised but at pH 5.2 and 6.5 the yields of corresponding methyl ketones rose to a maximum after about 8 days and then decreased.

In the absence of ammonium ion at pH 5.2, all triglycerides but trilaurin were oxidised to the corresponding methyl ketones to about the same extent. At pH 6.5 the highest yield of ketone was formed from trioctanoin. In all cases the yield of ketone continued to increase after 34 days (Table 41). In another experiment, with a different batch of spores, ketone formation increased slowly for 50 days (Fig. 28).

At all pH's, in the presence and absence of ammonium ion, trilaurin was not oxidised by spores to 2-undecanone to any significant extent.

Methyl ketone formation in a medium weakly buffered at pH 5.2 and containing ammonium ion

The medium used in the previous experiment at pH 5.2 was fairly heavily buffered (100 μ moles phosphate per ml). The effect of using a medium containing ammonium ion but

Table 41. The effect of pH on methyl ketone formation from triglycerides by spores in the absence and presence of ammonium ion (25 μ moles/ml)

pH	Substrate*	NH ₄ ⁺	Mycelial Growth after 8 days	Percentage yield of methyl ketone Days of incubation				
				1	2	8	20	34
2.5	Tributylin	+	Slight	Tr	0.2	0.2	0.6	6.1
	Trihexanoin	+	"	Tr	0.4	4.8	4.5	4.8
	Trioctanoin	+	"	3.3	3.9	3.1	3.1	3.3
	Trilaurin	+	None	Nil	Tr	Tr	Tr	Tr
5.2	Tributylin	+	Extensive	Nil	1.1	11.0	6.7	1.7
	Trihexanoin	+	"	3.0	0.3	0.3	Nil	Nil
	Trioctanoin	+	"	3.3	0.4	0.3	Nil	Nil
	Trilaurin	+	"	Tr	Tr	Tr	Tr	Tr
6.5	Tributylin	+	Extensive	Nil	2.2	8.8	2.2	0.8
	Trihexanoin	+	"	0.3	23.7	23.8	4.1	3.0
	Trioctanoin	+	"	1.9	1.9	10.0	4.3	0.9
	Trilaurin	+	"	Tr	Tr	Tr	Tr	Tr
5.2	Tributylin	-	Slight	Nil	1.7	1.8	5.9	14.0
	Trihexanoin	-	"	1.5	9.0	9.2	10.0	13.0
	Trioctanoin	-	None	2.4	9.6	9.4	10.1	12.0
	Trilaurin	-	"	Tr	Tr	Tr	Nil	Nil
6.5	Tributylin	-	None	0.1	0.1	0.2	0.2	0.3
	Trihexanoin	-	"	Tr	0.2	1.8	10.8	16.0
	Trioctanoin	-	"	3.5	12.0	21.1	22.1	23.0
	Trilaurin	-	"	Tr	Tr	0.7	1.2	1.5

Tr = trace = less than 0.05% yield

* 0.1 ml of each triglyceride added to 30 ml medium, each ml containing 100 μ moles phosphate buffer and approximately 1×10^6 spores, 14 days old.

less buffer (30 μ moles phosphate per ml) was also determined. As expected tributyrin and trihexanoin were oxidised to the corresponding methyl ketones in greater amounts than when the pH was kept at pH 5.0 - pH 5.2. There was a lag period of 6 days during which time the yield of ketones from tributyrin and trihexanoin was low (Fig. 32). In this time the pH of the medium fell to pH 3.5 and thereafter the yield of ketone rapidly increased. In 20 days 22% of the tributyrin was oxidised to acetone and 15% of the trihexanoin to 2-pentanone.

Free fatty acids in incubation media

No significant amounts of free fatty acids were detected in any of the media examined at any stage of the incubation. Since appreciable breakdown of the triglycerides to methyl ketones occurred, even at pH's < 3 at which lipolytic activity by fungi had never been reported, it can only be assumed that either the spores metabolised the fatty acids as soon as they were liberated or that the methyl ketones were formed by a pathway not involving free fatty acid.

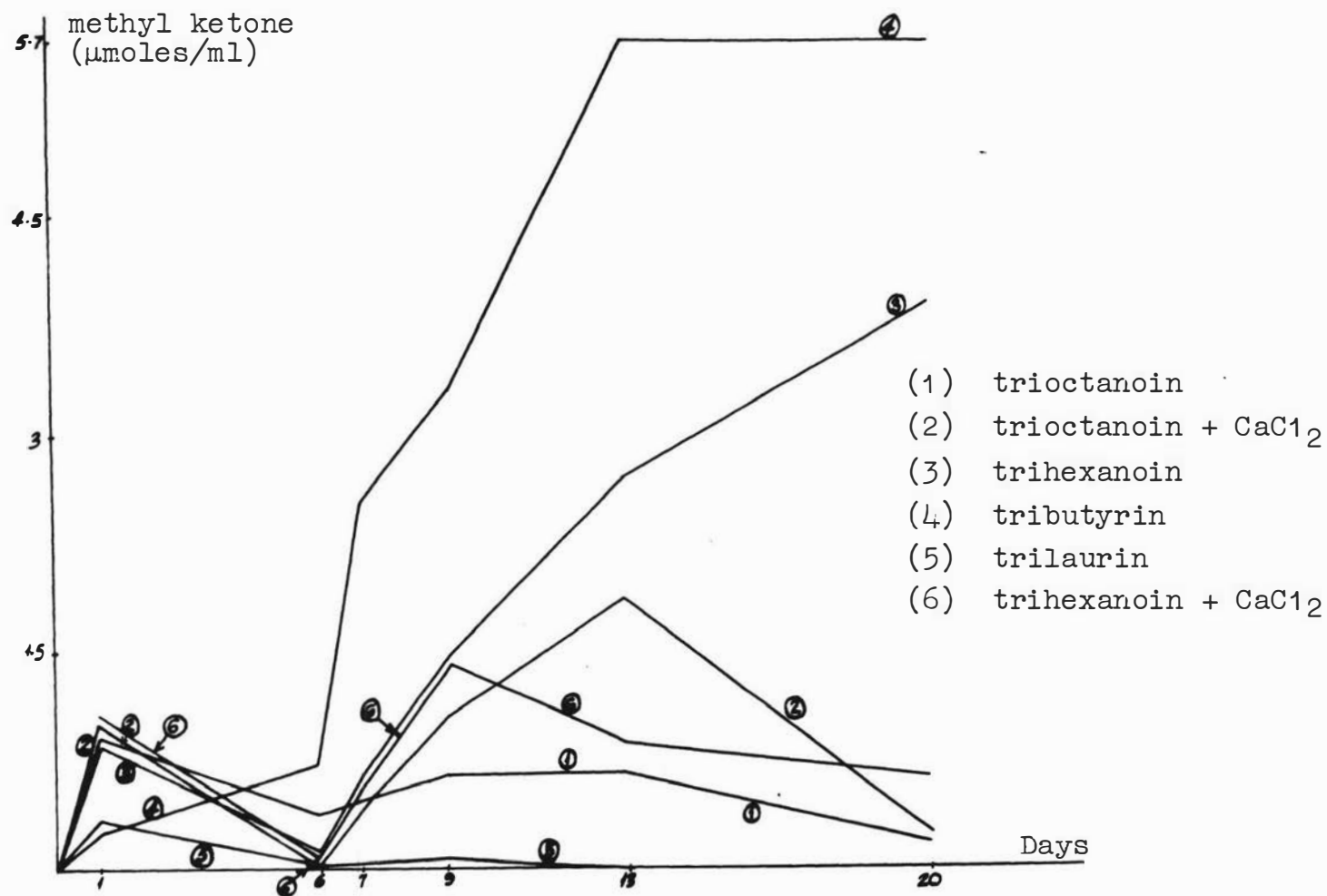


Fig.32. The formation of methyl ketones from triglycerides by 14 day old spores in a medium weakly buffered initially at pH 5.2. Each flask contained 0.1 ml triglyceride, about 1×10^6 spores/ml, phosphate buffer (30 $\mu\text{moles/ml}$) and ammonium sulphate (25 $\mu\text{moles/ml}$). Flasks 2 and 6 contained 0.2% CaCl_2 .

THE OXIDATION OF FATTY ACIDS BY CELL FREE EXTRACTS
PREPARED FROM MYCELIUM

Metabolic studies with mycelium were complicated by the high rate of endogenous respiratory activity of the intact cells. It was considered desirable as a concurrent approach to study fatty acid oxidation by cell free extracts. Such preparations eliminate permeability effects as well as high endogenous respiration but, as pointed out by Casida & Knight (1954), introduce the problem of phosphatase action on co-factors. They found that of the dehydrogenases of the T.C.A. cycle isolated from P. chrysogenum only the succinic dehydrogenase, which required no phosphorylated co-factors, functioned in cell-free preparations. Although fatty acid dehydrogenases (Mukherjee, 1951; Franke & Heinen, 1958) and T.C.A. cycle dehydrogenases (Casida & Knight, 1954) had been isolated from fungi, a complete fatty acid oxidising system had not been prepared. Such systems isolated from rat liver were shown by Munoz & Leloir (1943) to need Mg^{++} , PO_4^{3-} , ATP and cytochrome C. They also recognised that fatty acid oxidation could not be sustained unless some citric acid cycle oxidation was proceeding. The end product of fatty acid oxidation is acetyl CoA, which undergoes no further change unless oxalacetate is present. The conversion of the fatty acid to its thiol ester with Coenzyme A is an ATP -dependent reaction and thus the supply both of ATP and of oxalacetate is guaranteed by the operation of the citric acid cycle.

Cantino (1953) demonstrated that succinic acid dehydrogenase activity could be obtained from aquatic phycomycetes by simple disintegration of the mycelium

in a mechanical blender. Casida & Knight (1954) and Godzeski & Stone (1955) obtained TCA cycle dehydrogenases by grinding the mycelium of P. chrysogenum with sand. Some investigators have used methods of obtaining enzyme preparations which contain also the enzymes otherwise left behind in the cell fragments. Thus Knight (1948) successfully obtained acetone powders with amino acid oxidase activity from P. chrysogenum and Karrer & Haab (1948) found that dried mycelial powders from various Penicillia were capable of decarboxylating β -keto acids to the corresponding methyl ketone.

Preparation of cell free extracts

Attempts to obtain preparations capable of oxidising octanoic acid, using the above methods of Karrer & Haab (1948) Knight (1948) and Godzeski & Stone (1955), were unsuccessful. In other experiments mycelium was washed with dilute ethanol or acetone (10-12%) to increase the permeability of the cell walls, but such cells were completely inactive.

Extracts with slight activity were obtained by disintegration of the cells with a Polytron homogeniser. More active extracts were obtained by drastic disintegration with a Hughes Press. In this technique the mycelium was blended in a modified Krebs-Ringer buffer (Krebs & Eggleston, 194C), 5ml of the suspension being put through the press. This applied instantaneously pressure of about 15 tons per square inch. The cells were pre-cooled to -20° to -30° , the ice crystals formed serving as an abrasive. The frozen extract and broken

cells were allowed to warm, resuspended using the Polytron homogeniser and centrifuged at 1000 g for 20 min. The supernatant was decanted, leaving behind uncrushed cells and cell debris. The extracts remained active for at least 24 hours in a refrigerator.

Factors affecting the activity of cell-free extracts

(1) Composition of buffer solution. Since other workers, e.g. Lehninger (1945), have found the composition of the buffer used in the preparation of cell free extracts to be of great importance, different phosphate buffers were used to obtain extracts from the Hughes Press. Little difference in activity was however observed (Fig. 33) and in most experiments Krebs-Ringer buffer, modified to give a pH of approximately 6.0, was used.

(2) Rate of spinning down of debris. An active extract was not obtained after blending (or after using the Hughes Press) if the suspension was centrifuged at speeds greater than 3000 g. Since the supernatants obtained by centrifuging at 1000 g. for 20 min. occasionally contained spores, they were decanted and recentrifuged at 2000 g for a further 10 min.

Succinic dehydrogenase activity. In preliminary experiments it was found that a correlation existed between the ability of a cell free extract to oxidise fatty acids and its succinic dehydrogenase activity. The latter was therefore routinely checked in a Thunberg

tube to ensure that at least part of the respiratory system was present.

1 ml of supernatant was added to a mixture of 2 ml 0.2M sodium succinate, 2 ml 0.1M phosphate buffer (pH 6.4) and 0.5 ml 0.02% 2,6-dichlorophenol-indophenol. With extracts from the Hughes Press the colour disappeared within 30 minutes at 30°. Extracts obtained by homogenisation took longer (up to 45 minutes) to decolourise the dye.

Oxidation of octanoic acid by cell-free extracts from the Hughes Press

The pattern of oxygen uptake during the oxidation of octanoic acid by the extracts was usually similar to that shown in Fig. 33. Oxygen uptake increased rapidly after a lag period of 1 to 2 hours and was normally complete after 6 hours. Little difference in activity was observed in the presence of added magnesium ion. On one occasion addition of 0.15 μ moles magnesium ion eliminated the lag phase, but this effect could not be repeated with other cell free preparations. The enzyme preparations were only able to oxidise low concentrations of octanoic acid (less than 1 μ mole/ml). Thus one extract was able to oxidise 0.5 μ moles C_8 acid but not 2 μ moles. Such results are similar to those of Stumpf & Barber (1956) who found that high concentrations of butyric acid (5 and 10 x 10⁻³M) inhibited the respiratory system of the peanut mitochondrion although lower concentrations were readily oxidised.

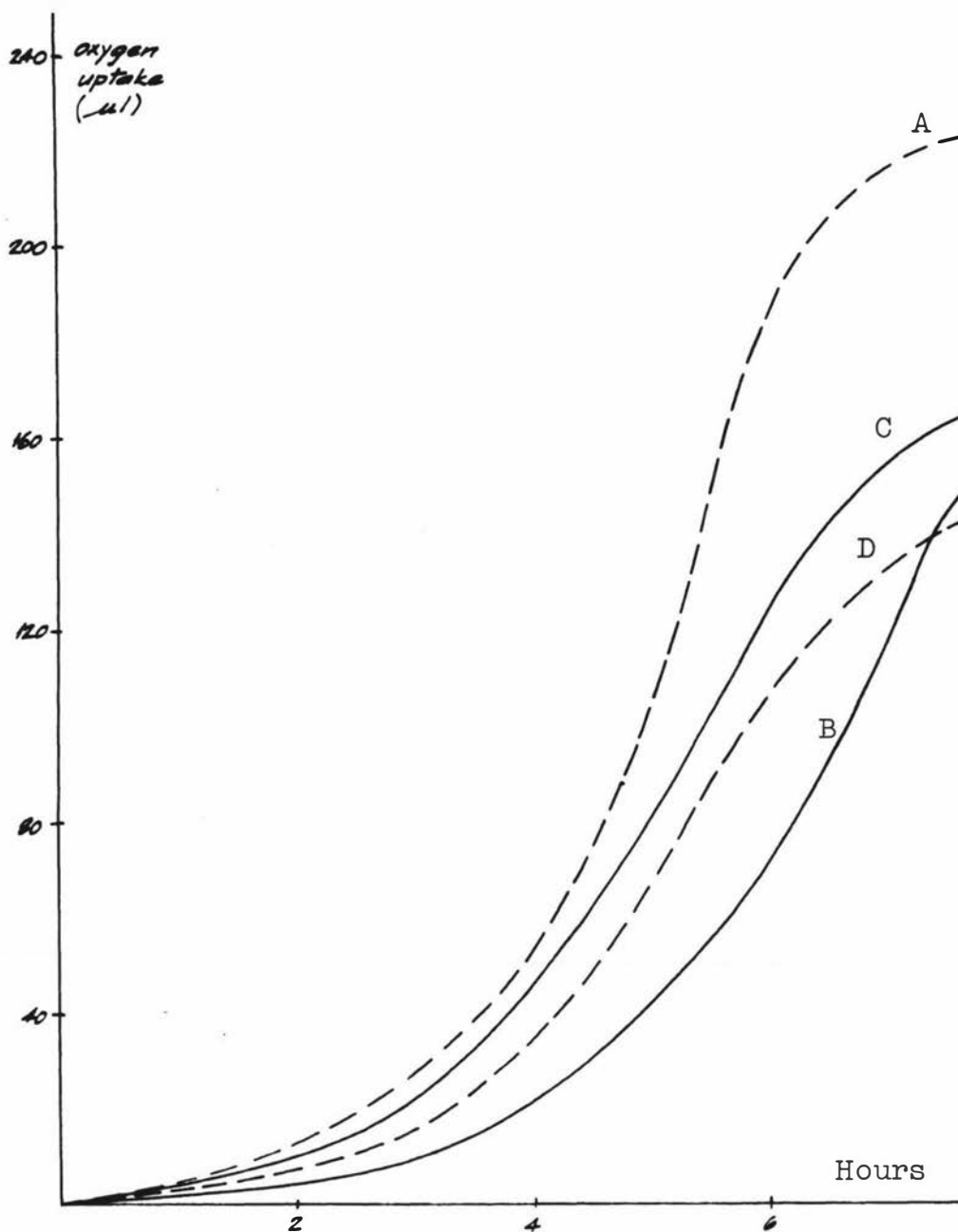


Fig.33. The oxidation of octanoic acid at pH 6.0 by cell free preparations extracted from 4 day old mycelium in the Hughes Press with modified Krebs Ringer buffer (flasks A and B) and with M/60 potassium phosphate buffer (pH 6.0) + M/15 KCl (flasks C and D). Flask A contained 1 μ mole and flasks B, C and D 0.75 μ moles octanoic acid. 0.5 μ moles Mg^{++} were added to flask D.

The possibility was considered that the oxygen uptakes observed were due to contamination of the extracts by bacteria or spores but none was however detectable under the microscope. Spores also invariably partially oxidised added acid substrate to methyl ketone yet none was isolated when cell free extracts were incubated with fatty acids.

Addition of co-factors, inhibitors and TCA cycle intermediates

Attempts to eliminate the lag phase by supplementing the cell-free preparations with 10^{-3} M adenosine triphosphate (ATP) were not successful. Indeed in higher concentrations (10^{-2} M) ATP was inhibitory. McGilvery (1957) also observed the toxic effect of commercial ATP samples and suggested that heavy metal contamination was responsible. In an attempt to overcome possible heavy metal effects, EDTA (ethylene diaminetetracetic acid) was added (0.25 ml of a 1% solution) with ATP but this did not enhance oxygen uptake. Glucose, added as a potential source of ATP, was not oxidised by the extracts.

According to Hunter (1955) the addition of sodium fluoride (0.01M) to reduce ATP-ase and phosphatase activity was essential in broken cell preparations. It tends however also to inhibit Mg^{2+} dependent enzymes and it was found in this investigation that oxygen uptake was faster in extracts not supplemented with NaF.

During the preparation of cell-free extracts the terminal respiratory system is usually disrupted

(Cochrane, 1958) but neither cytochrome C (10^{-4} M) nor methylene blue (10^{-3} M) enhanced oxygen uptake. Methylene blue was intended to serve as an artificial hydrogen carrier in order to replace the still unknown terminal electron system.

Other supplements which had no effect upon the oxidation of octanoic acid by the cell-free extracts were 10^{-3} M adenosine diphosphate (ADP), 10^{-3} M diphosphopyridinenucleotide (DPN), 10^{-6} M flavin adenine dinucleotide (FAD) and TCA cycle intermediates (1 μ mole each of succinate, oxalacetate and fumarate).

Activity of mycelial debris from the Hughes Press

In order to determine to what extent, if any, the oxidative enzymes were destroyed by homogenisation or by passage through the Hughes Press, the ability of debris after such treatments to oxidise fatty acids was compared with oxidation by whole cells and blended suspensions. Debris for this comparison was obtained as follows:

- (a) Mycelium was blended at top speed in phosphate buffer, pH 6.0, for 2 min, the debris being centrifuged for 20 min. at 1000 g.
- (b) Mycelium was put through the Hughes press, the debris being washed in buffer and the suspension centrifuged at 1000 g for 20 min. The debris was again washed in buffer and re-centrifuged for a further 20 min.

The rate of oxygen uptake by the debris from the Hughes press in the presence of octanoic acid was about

25% of that by the whole mycelium. On the other hand oxygen uptake by debris obtained after blending mycelium at high speed was slightly higher than that of whole cells. Endogenous respiration of debris from the Hughes Press was also very much lower than with whole cells (Table 4.2). It would appear that certain enzymes or cofactors are common to both endogenous respiration and the oxidation of exogenous fatty acid. This was confirmed by combining the debris from the Hughes Press with the supernatant obtained by centrifuging. The resultant suspension was still unable to oxidise the fatty acid at the normal rate showing that partial damage had been done to the oxidising system.

Since no cell-free extract examined possessed the ability to form methyl ketones from fatty acids, the finding that the debris from the Hughes Press was as active as whole cells in forming methyl ketones was not surprising. This is also in agreement with Karrer & Haab (1948) who concluded that the β -keto acid decarboxylases of *Penicillia* were bound very firmly to the cell.

Table 42. A comparison of the ability of mycelial whole cells and debris to oxidise octanoic acid

	Oxygen uptake (μ l)	Yield 2-heptanone (μ moles)	Time for complete oxidation (hours)
<u>Whole mycelium</u>			
Endogenous	140	-	-
1 μ mole C ₈ acid	294	0.16	1.75
2 μ moles " "	440	0.80	2.0
<u>Debris from vigorously blended mycelium</u>			
Endogenous	130	-	-
1 μ mole C ₈ acid	308	0.15	1.5
2 μ moles " "	471	0.70	1.75
<u>Debris from Hughes Press</u>			
Endogenous	51	-	-
1 μ mole C ₈ acid	116	0.24	3.5
2 μ moles " "	156	0.77	3.5
<u>Suspension from Hughes Press</u>			
Endogenous	88	-	-
1 μ mole C ₈ acid	207	0.20	3.5
2 μ moles " "	260	0.65	3.5

(Each Warburg flask contained 10 mg mycelium or debris, 50 μ moles phosphate buffer, pH 6.0.)

DISCUSSION OF

PART III

Considerable emphasis has been placed recently on the biochemical uniformity of biological systems and yet the oxidation of fatty acids to the corresponding methyl ketones with one carbon atom less has only been reported with fungi. Many of the dissimilation products of fungal metabolism are never present in more than traces in other biological systems (animal tissues, bacteria, yeast), being formed and transformed so quickly that they never accumulate. The mechanism of formation of methyl ketones, i.e. deacylation followed by decarboxylation, has however an analogy in animal tissues, the ketogenic activity of animals on high fat diets for instance being well known (Campbell & Best, 1956). Similarly, isolated liver mitochondria have been shown to accumulate acetoacetate almost quantitatively during fatty acid oxidation in the presence of malonate. This accumulation appears to be a consequence of two circumstances - the deacylation of acetoacetyl CoA to acetoacetate and the absence in liver of a thiokinase for converting acetoacetate back to acetoacetyl CoA (Green, 1963).

The demonstration by Butts, Cutler, Hallman & Deuel (1935) that the excretion of acetone bodies from rats was significantly greater after the administration of C_8 acid than other acids was similar to the finding in this investigation that some acids, particularly C_8 acid, were more readily oxidised than others to methyl ketones. Kennedy & Lehninger (1950) found a fundamental difference in the metabolism of short and long chain fatty acids by isolated rat liver mitochondria. Those with an even number of carbon atoms $> C_{12}$ gave CO_2 as the main

product and those with an even number $< C_{12}$ gave primarily acetoacetate. These findings were similar to those in the present study where only the C_4 to C_{12} acids were oxidised to the corresponding methyl ketone. The pattern of oxidation of "even and odd numbered" fatty acids in this investigation was identical, in agreement with the conclusion of Mii & Green (1954) that isolated enzymes of the fatty acid oxidation cycle from liver made no distinction between the odd and even numbered acids.

Although the higher (C_{14} - C_{18}) fatty acids were not oxidised by fungi to the corresponding methyl ketones, small quantities of acetone were formed, a similar situation occurring under certain conditions in animal tissues such as the liver. The presence of acetoacetic decarboxylase in fungi was not unexpected since the enzyme has been found, for instance, in Clostridium acetobutylicum (Wood, 1961).

The mechanism of β -oxidation of fatty acids by fungi has some unexplained facets. The oxidation of octanoic acid by the classical pathway (Fig. 34) would allow the formation of β -keto octanoyl CoA, β -keto hexanoyl CoA and β -keto butanoyl CoA and the subsequent formation of the corresponding methyl ketones. However only 2-heptanone was detected (when octanoic acid was oxidised) although the same batch of mycelium was shown to be quite capable of oxidising hexanoic acid to β -keto hexanoyl CoA since 2-pentanone was formed. Since the free -SH and $-NH_2$ groups of sulphhydryl enzymes or cofactors such as glutathione might be expected to add readily across the active β -keto group of the $-CO.CH_2.COOH$ moiety, it is possible that the first β -keto acid formed is toxic to some enzymes of the fatty acid oxidation

cycle. The inhibition of the action of thiolase upon the first β -ketoacyl Coenzyme A formed would explain the subsequent detection of only one methyl ketone.

Franke, Platzeck & Eichhorn (1961) concluded from their studies that the β -keto acid decarboxylases were constitutive enzymes. They considered that the removal of β -keto acids was important to fungal cells and that this property (to give methyl ketones) served as a non-specific inhibitor against sensitive competitors for the "Lebensraum" during germination. The present investigation however throws some doubt on this conclusion since the rate of methyl ketone formation (Figs. 10a and 10b) suggests the synthesis of adaptive decarboxylases after a lag period of 1 to 2 hours. The accumulation of a toxic β -keto acid in the cells might be expected to induce the synthesis of a β -keto acid decarboxylase, with subsequent formation of methyl ketone, since this would aid the removal of a toxic metabolite.

An hypothesis based upon the possible toxicity of the C_6 to C_{12} β -keto acids does explain a number of the experimental results:

1. Only one methyl ketone and thus presumably only one β -keto acid was formed.
2. The most toxic acids, C_{10} , C_{11} and C_{12} acids, were those which gave the least amount of methyl ketone.
3. Concentrations of the C_8 to C_{12} fatty acids that markedly inhibited the respiration of mycelium were nevertheless oxidised to considerable quantities of the corresponding methyl ketone. It would appear that the enzymes responsible for the oxidation of the fatty acids to β -keto acids are less sensitive to high concentrations

of the acids than those enzymes responsible for the further oxidation of the β -keto acids.

4. Cells shaken in a low concentration (2 to 4 μ moles) of C_{10} acid, and then thoroughly washed, were subsequently able to oxidise C_8 acid to 2-heptanone but not, as shown by the use of $2-C^{14}$ octanoic acid, to carbon dioxide and water. If the C_{10} acid almost completely inhibited the enzymes responsible for the oxidation of β -keto acids, C_8 β -keto acid might then be expected to accumulate, resulting in a greater formation of 2-heptanone.

5. Increased yields of 2-heptanone were also obtained if the C_{10} acid were added after the C_8 acid, although the yield was lower than with the reverse order. If the C_8 had already been slowly oxidised by the cell, up to and including the C_8 β -keto acid, the addition of the C_{10} acid presumably prevented its further oxidation and hence decarboxylation occurred to a greater extent.

The toxicity of octanoic acid at pH 2.6 was shown to be reversible since raising the pH to 5.5 with alkali allowed the mycelium to rapidly oxidise the acid. The activity was not restored immediately, lag period being dependent upon the concentration of octanoic acid initially added. The enzymes do not themselves appear to be pH dependent since they readily oxidised non-toxic concentrations of C_8 acid (e.g., 1 μ mole) at pH 2.5.

In direct contrast to the numerous experiments carried out in this investigation, Stadtman & Barker (1949) found that the rate of butyrate oxidation by Clostridium kluyveri was unaffected by the addition of a toxic concentration of C_{10} acid. They concluded, from one experiment only at an unspecified pH, that the bacterial enzymes were specific for fatty acids of eight or fewer carbon atoms.

These studies of the metabolism of fatty acids by fungi have allowed inferences to be made concerning an intermediate (the β -keto acid) which are not possible in other biological systems. For example, if the suggestion of Nieman (1954) that fatty acids are toxic because of their adsorption on the cell wall were true, then increased inhibition of oxidation with increasing chain length as well as with increasing concentration would be strictly physical. Since cells shaken in a toxic concentration of C_{10} acid, and then thoroughly washed with pH 7.0 buffer, were unable subsequently to oxidise octanoic acid to CO_2 , the toxic action of the C_{10} acid must be intracellular. Similarly the fact that the $C_6 - C_{12}$ fatty acids were able to inhibit endogenous respiration of mycelium at pH's below 6.0 and yet be oxidised to methyl ketones suggests that the acids must actually enter the cell, supporting the hypothesis of Cochrane (1958) that the primary inhibitory effect of toxic compounds occurs after they have penetrated the cell wall.

It has been generally observed (e.g. Cochrane, 1958) that toxicity does not continue to increase above a certain chain length, the actual length depending on the homologous series and on the test organism. The present investigation confirmed this but also added the new dimension of pH. The chain length of the most toxic fatty acid increased with pH, being C_{10} acid at pH 2.5 and C_{12} acid at pH's 5.2 and 6.0. At pH 6.8 none of the acids from C_4 to C_{18} inhibited oxygen uptake but C_{14} acid was the most toxic at pH 8.0. It seems likely however that this toxicity at pH 8.0 is probably a detergent effect on the surface of the mycelium and distinct from the toxicity observed at pH 6.0 and below. The relationship between

optimum chain length and the toxic action of fatty acids is thus very dependent upon pH. This conclusion allows a ready explanation of the apparent contradiction between the results of Rolinson (1955) who reported that C₈ and C₁₀ acids were toxic to P. chrysogenum at pH 6.3 and those of Girolami & Knight (1955) who found that at pH 7.3 these 2 acids stimulated oxygen uptake by P. roqueforti to a greater extent than other acids. Similarly in this investigation the C₉ and C₁₀ acids more actively inhibited the respiration of mycelium at pH 2.5 than other acids but were the most rapidly oxidised at pH 8.0. Conversely those acids (C₃, C₄, C₅ and C₁₄ acids) most readily oxidised at pH 2.5 were those which were the least rapidly oxidised at pH 8.0.

The observed variation with pH of the inhibitory effect of fatty acids upon oxygen uptake by mycelium is most easily explained by assuming that only undissociated molecules of fatty acids are oxidised and that in high concentrations they or their oxidation products are toxic. Permeability effects prevent dissociated fatty acids from penetrating the cell wall and being oxidised. The relatively low concentrations of undissociated acid present above pH 7.0 would explain the slower oxidation of acids obtained at pH 8.0 in this investigation.

In general four patterns of fatty acid oxidation by mycelium were distinguishable : (a) The short chain acids from C₁ to C₆ were readily oxidised only at pH's below 5.5, the C₅ and C₆ acids giving high yields (up to 75%) of the corresponding methyl ketone. Acetone formation was never observed (apart from butyric acid). (b) The C₇ and C₈ acids were toxic below pH 5.2 but were readily oxidised above this pH. Both formed the corresponding ketones readily, up to 70% in the pH range

5.0 to 6.5. No acetone formation was detected.

(c) The $C_9 - C_{12}$ acids were toxic below pH 6.0 but were readily oxidised above this pH. Only small amounts of the corresponding methyl ketone (optimum about 25% at pH 8.0) were detected. Below pH 6.0 approximately equal proportions of acetone and the corresponding methyl ketone were formed.

(d) The long chain fatty acids ($C_{14} - C_{18}$) were oxidised only very slowly in acid conditions and even less readily in alkaline. They formed no corresponding methyl ketone but, at pH 5.2 and below, very small amounts of acetone were detected.

Differences in oxygen uptake and methyl ketone formation were observed with the same concentration of acid but with different batches of mycelium. It was, however, possible in all the experiments to show significant trends and no results were obtained significantly at variance with each other. The acid which was oxidised to methyl ketone in high yield most consistently over a wide range of pH was C_8 acid. This was contrary to the finding of Franke & Heinen (1958) that fungi, including P. roqueforti, oxidised C_4 and C_{12} acids most readily to the corresponding methyl ketones. The experimental conditions described by these workers were simulated but the maximum yield of ketone was still obtained from C_8 acid. They also reported the oxidation of C_{14} acid to 2-tridecanone in higher yields than that of 2-heptanone from octanoic acid. No 2-tridecanone at all was detected from C_{14} acid with the two strains of P. roqueforti used in this investigation, although small amounts of acetone were obtained.

The toxicity of any particular fatty acid to mycelium was markedly altered when the cells were pre-starved by shaking them in phosphate buffer for at least 24 hours. The respiration of fresh mycelium was almost completely inhibited by 3 μ moles octanoic acid at pH 2.5 whereas the same mycelium shaken for 48 hours in buffer was able to oxidise the acid rapidly. The cells used in this study were grown with glucose which is known to inhibit the synthesis of many constitutive enzymes of fungi (Magasanik 1957). It has been suggested (Neidhardt, 1960; Magasanik, 1961) that this repression by glucose results from the high intracellular concentration of intermediates of glucose catabolism which prevents the synthesis of those enzymes whose products would only augment the already large metabolic pools. When cells are shaken in buffer it is possible that these intermediates are used for respiratory purposes, thus removing the repression governing the synthesis of enzymes whose function is to supply energy, in this case by the oxidation of fatty acids.

The fact that there was no definite increase in respiration in this investigation when 2,4 dinitrophenol was added shows that little oxidative assimilation occurred between pH 5.5 and pH 6.5. This was confirmed by the virtually quantitative oxygen balances obtained in many experiments. These results were unusual since fungi in general are characterised by their high oxidative assimilation (Foster, 1949). It is possible, however, that fatty acids themselves (and in particular long chain fatty acids) are to some extent "decouplers" since Borst, Loos, Christ & Slater (1962) have shown that long-chain fatty acids can stimulate the latent

ATP-ase activity of fresh rat-liver mitochondria in presence of Mg^{++} . It is interesting to note that the fatty acids in their experiments were scarcely stimulatory above pH 8 and in the present study also the oxygen uptake and methyl ketone formation with saturated fatty acids were minimal at pH 8.

The lipid content of *Penicillia* is generally very high and can constitute up to 50% of the dry mycelium. The free fatty acid content ranges from 8 to 70% of the fat (Cochrane, 1958), and this may be related to the ease with which both spores and mycelium oxidised fatty acids in this study. The general belief that the normal metabolism of spores is a fat metabolism during the first stages of germination was supported by Farkas & Ledingham (1959) who found that resting rust uredospores were able to utilise fatty acids about 10 to 20 times more readily than carbohydrates and amino acids. They observed that a 50 to 100% increase in oxygen uptake in the presence of fatty acids was further stimulated by the presence of carbon dioxide, which was shown to be incorporated into the Krebs cycle. Similarly in the present investigation the formation of methyl ketone was stimulated by the small amounts of metabolic CO_2 evolved.

The contention of Berridge, Hiscox & Zielinska (1953) and Gehrig & Knight (1963) that spores but not mycelium are able to metabolise fatty acids to methyl ketones was not confirmed. Gehrig & Knight used relatively high concentrations of C_8 acid (20 μ moles/3ml) which were conceivably toxic to the mycelium used but not to the spores. They detected no methyl ketones when low concentrations (not specified) of octanoic acid were oxidised by spores. This inability to form methyl ketones would appear to ^{be} specific to the strain of

P. roqueforti they used since the 2 strains employed in this investigation, were both able to oxidise between 40 and 75% of added octanoic acid to 2-heptanone, even with concentrations as low as 0.25 μ moles/ml.

In this investigation there did not appear to be a sharp dividing line between the metabolic activity, with respect to fatty acids, of spores and mycelium. The results obtained support the conclusion of Cochrane (1958) that there is as yet no reason to believe that spore respiration differs fundamentally from that of mycelium. Mycelium oxidised fatty acids rapidly giving varying amounts of methyl ketone but considerably more CO_2 than spores. The latent ability of mycelium to form ketones was shown by pre-incubation with a low concentration of C_{10} acid when the yield of 2-heptanone from octanoic acid subsequently added was increased greatly. In general ungerminated spores formed higher amounts of methyl ketones than mycelium but showed also a slight but definite ability to form CO_2 from octanoic acid, which was greatly enhanced by incubating the spores for a short period with ammonium ion.

CO_2 formation at the expense of methyl ketone formation in the presence of ammonium ion may be due to the drawing off of intermediates for the synthesis of amino acids. If, for example, glutamic acid were formed from α -keto glutarate, an increased formation of glutamic acid, and its subsequent removal for protein synthesis, might accelerate the entire sequence of reactions leading from octanoic acid via acetyl CoA to intermediates of the tricarboxylic acid cycle. Such a mechanism for the alternative utilisation of β -keto acid formed from fatty acid could account for both the increased formation of labelled CO_2 and decreased

formation of 2-heptanone from 2-C¹⁴ octanoic acid in the presence of ammonium ion.

The low rate of conversion of 2-C¹⁴ octanoic acid to ¹⁴CO₂ by spores (Table 25) may be a result of the assimilation of part of the utilised carbon into reserve material of the cell. Clifton (1963) put forward the hypothesis that endogenous pools are a major source of energy in glucose metabolism and that exogenous substrate in part at least serves to replenish the supply of endogenous reserves. This is also the opinion of Blumenthal (1963) who considered it likely that there is an interplay between exogenous and endogenous substrates and that the question as to when the substrate ceases to be exogenous and becomes endogenous is difficult to define. If the newly-assimilated fatty acid "pooled" rapidly with the endogenous reserve, the recovery of radioactive carbon as ¹⁴CO₂ might be expected to be low since the carbon of the added fatty acid would serve in part to replenish the endogenous reserves being oxidised.

The 2-heptanone formed during the oxidation of 1-C¹⁴ octanoic acid was not radioactive showing that the carbon atom from the carboxyl group was lost during the decarboxylation. 2-heptanone from 2-C¹⁴ octanoic acid was radioactive, its specific activity being almost identical to that of the original acid. Both these findings afford strong evidence for a β -oxidation mechanism.

The significance of a T.C.A. cycle in fungal respiration has however yet to be clearly established, large differences in the ability of fungi to oxidise TCA intermediates apparently existing even between strains of the same species (Ajl, 1951). Although evidence is

generally in favour of a TCA cycle operating, Cleland & Johnson (1953) have claimed findings to the contrary.

It was therefore of interest to examine, as a function of time, the extent of labelling of the respiratory CO_2 from octanoic acid-1- C^{14} and octanoic acid-2- C^{14} . The results summarised in Table 25 support the hypothesis that the mycelium was oxidising fatty acids primarily through the β -oxidative pathway, since the rate of appearance of ^{14}C in respiratory carbon dioxide was 1.5 to 3 times greater for 1- C^{14} octanoic acid than for 2- C^{14} octanoic acid. As citrate is handled assymmetrically by the enzymes of the TCA cycle, at least 3 turns of the cycle are required before an even numbered carbon atom of a fatty acid (the methyl group of the active acetate unit) appears in the respiratory CO_2 . On the other hand, an odd numbered carbon atom of a fatty acid (the carboxyl group of the active acetate unit) appears in respiratory CO_2 as soon as the second turn of the cycle is completed (Katz & Chaikoff, 1955).

The evidence indicated a rather slow movement of compounds through the TCA cycle under the experimental conditions. Since methyl ketone formation was also slow, none being detected until 1 to 2 hours after addition of acid, it is possible that the rate of fatty acid oxidation is determined by the initial reactions leading to β -keto acid.

High concentrations of triglycerides were readily oxidised by spores to methyl ketones when equivalent concentrations of the free acid (up to 66 $\mu\text{moles/ml}$) were barely oxidised. It would appear that the slow hydrolysis of the triglycerides resulted in the incorporation of the fatty acids, at a sufficiently low rate to prevent a toxic concentration of the acid (or an oxidation product such

as β -keto acid) accumulating but which allowed high methyl ketone formation. With increasing concentration of triglyceride the amount of free acid available presumably reached a point where it was toxic to the cell, the yield of methyl ketone being correspondingly very low (Table 40).

No free fatty acids accumulated in the medium during the incubation which indicated that the lipolytic step was rate governing and not the incorporation of the acid into the spores and its subsequent oxidation to methyl ketone. Attempts to increase lipase activity by the addition of ammonium ion usually resulted in a much greater yield of methyl ketone initially but the extensive mycelium also formed was generally accompanied by a slow decrease in the amount of methyl ketone detected (Fig. 29). More consistent results were obtained by using spores without an external source of nitrogen, the rates of formation of methyl ketone being lower but eventually giving higher yields. Lipases in spores of P. roqueforti do not appear to be adaptive enzymes since, unlike bacterial lipases, they were produced in media devoid of materials that ordinarily serve as substrates for lipase formation.

Weinstein & Wynne (1936) found that $3.7 \times 10^{-3}M$ 2-octanone (i.e. $3.7 \mu\text{moles/ml}$) decreased the rate of hydrolysis by pancreatic lipase by 80% in 15 minutes. Since in this investigation up to $17 \mu\text{moles}$ 2-heptanone per ml were formed, it seems probable that the very slow formation of methyl ketone was due to the inhibitory effect of the ketones themselves on the lipases. This might account for the finding that emulsification or the addition of known lipase activators such as calcium salts had no consistent effect on methyl ketone formation from triglycerides.

This investigation supported the suggestion of Foster (1947) that accumulated products of fungal metabolism (in this case mainly 2-heptanone) are not further metabolised until the organism exhausts the more easily attacked and preferred energy source. Spores were found to be as capable as mycelium of metabolising methyl ketones, contrary to the hypothesis of Berridge et al. (1953). Methyl carbinols were not detected in significant amounts as products of the metabolism of methyl ketones, evidence being obtained, on the contrary, that the carbinols were possibly precursors of the ketones during the oxidation of fatty acids by spores. Thus 2-heptanol was initially present in greater concentration than 2-heptanone during the oxidation of octanoic acid by spores (Table 31) but only 2-heptanone was detected after 4 days. This may be related to the finding that mycelium oxidised 2-heptanol readily and virtually quantitatively to 2-heptanone whereas it was difficult to demonstrate that the reduction of 2-heptanone to 2-heptanol occurred at all. These results did not confirm the finding of Franke, Platzek & Eichhorn (1962) that methyl carbinols accumulate during the oxidation of fatty acids by fungal spores at the same rate as the yield of methyl ketone decreases. Substantial amounts of methyl carbinols accumulated however on incubating triglycerides with spores, up to 10% of trioctanoin being metabolised to 2-heptanol after 50 days. To what extent, if any, this was a consequence of the presence of free glycerol in the medium has yet to be determined.

Cell free extracts obtained from mycelium were able to oxidise low concentrations of octanoic acid (0.5 μ moles/ml or less) after a lag of up to 3 hours. A

possible explanation for the time lag might be the absence of some key intermediate, which only slowly accumulated during the oxidation. Thus, for instance, the absence of oxalacetate would prevent the formation of citric acid from acetyl CoA and the consequent release of Coenzyme A. The concentration of Coenzyme A in vivo is limited and must be regenerated for a repeat of the β -oxidation cycle. The supplementation of the cell free extracts with several coenzymes, known to be associated with fatty acid oxidation, or with TCA cycle intermediates were unable, however, to decrease the lag before oxidation started.

The activity of different preparations varied widely, which was perhaps to be expected in view of the number of factors involved in this system and the somewhat crude extraction methods used. No methyl ketones were detected after the oxidation of fatty acids by cell-free extracts but were formed when cell debris from the Hughes Press was used. This suggests that the β -keto acid decarboxylases were tightly bound to the cell walls.

REFERENCES

- Abraham, S., Matthes, K.J. & Chaikoff, I.L. (1961).
Biochim. Biophys. Acta. 49, 268.
- Achaya, K.T. & Hilditch, T.P. (1950). Proc. Roy. Soc.
(London). Ser. B. 137, 187.
- Acklin, O. (1929). Biochem Z. 204, 253.
- Ajl, S.J. (1951). Bacteriol. Revs. 15, 211.
- Alberts, A.W., Goldman, P. & Vagelos, P.R. (1963). J. biol.
Chem. 238, 557.
- Annison, E.F., Leng, R.A., Lindsay, D.B. & White, R.R.
(1963). Biochem. J. 88, 248.
- Archibald, R.M. (1946). J. biol. Chem. 165, 443.
- Babel, F.J. & Hammer, B.W. (1945). J. Dairy Sci. 28, 201.
- Badger, G.M. (1946). Nature, Lond., 158, 585.
- Barry, J.M., Bartley, W., Linzell, J.L. & Robinson, D.S.
(1963). Biochem. J. 89, 6.
- Bassett, E.W. & Harper, W.J. (1958). J. Dairy Sci. 41, 1206.
- Bavisotto, V.S., Rock, I.A. & Lesniewski, R.S. (1960).
J. Dairy Sci. 43, 849.
- Beevers, H. & Simon, E.W. (1949). Nature, Lond., 163, 408.

- Berridge, N.J., Hiscox, E.R. & Zielinska, M. (1953).
Int. Dairy Congr. II, 587.
- Bier, M. (1955). Methods in Enzymology Section III. Ed.
by Colowick, S.P. & Kaplan, N.O. New York : Academic
Press Inc.
- Black, A.L. Kleiber, M. & Brown, Alice M. (1961). J. biol.
Chem. 236, 2399.
- Bloomfield, D.K. & Bloch, K. (1960). J. biol. Chem. 235, 337.
- Blumenthal, H.J. (1963). Ann. N.Y. Acad. Sci. (1963).
102, 688.
- Boldingh, J. & Taylor, R.J. (1962). Nature, Lond., 194, 909.
- Borst, P., Loos, J.A., Christ, E.J. & Slater, E.C. (1962).
Biochim & Biophys. Acta. 62, 509.
- Bressler, R. & Wakil, S.J. (1962). J. biol. Chem. 237, 1441.
- Byrde, R.J.W. & Woodcock, D. (1956). Biochem. J. 64, 154.
- Campbell, J. & Best, C.H. (1956). Metabolism. 5, 95.
- Campbell Soup Company. (1961). Proceedings Flavour
Chemistry Symposium. Camden. New Jersey.
- Chiriboga, J. & Roy, D.N. (1962). Nature, Lond., 193, 684.
- Cleland, W.W. & Johnson, M.J. (1953). J. biol. Chem. 208,
679.

- Clifton, C.W. (1963). Ann. N.Y. Acad. Sci. (1963). 102, 655.
- Cochrane, V.W. & Peck, H.D. (1953). J. Bact. 65, 37.
- Cochrane, V.W. (1958) Physiology of Fungi. New York : John Wiley & Sons, Inc.
- Cochrane, V.W. (1960). Plant Pathology. Ed. J.G. Horsfall & A.E. Dimond. Vol. II. New York : Academic Press Inc.
- Cochrane, Jean C., Cochrane, V.W., Simon, F.G. & Spaeth, Jane. (1963). Phytopathology. 53, 1155.
- Coffman, J.R., Smith, D.E. & Andrews, J.S. (1960). Food Res. 25, 663.
- Coulter, S.T. (1938). J. Dairy Sci. 21, 273.
- Cowie, A.T. et. al. (1951). Biochem. J. 49, 610.
- Currie, J.N. (1914). J. Ag. Res. 2, 1.
- Cutchins, E.C., Doetsch, R.D. & Pelczar, M.J. (1952). J. Bact. 63, 269.
- Dacre, J.C. (1955). J. Dairy Res. 22, 219.
- Darby, R.T. & Goddard, D.R. (1950). Am. J. Bot. 37, 379.
- Darby, R.T. & Mandels, G.R. (1955). Plant Physiol. 30, 360.

- Day, E.A. & Keeney, M. (1958). J. Dairy Sci. 41, 718.
- Day, E.A., Bassette, R. & Keeney, M. (1960). J. Dairy Sci. 43, 463.
- Day, E.A. & Lillard, D.A. (1960). J. Dairy Sci. 43, 585.
- Derx, H.G. (1925). Proc. K. Akad. Wetensch. Amsterdam. 28, 2.
- Dils, R. & Popják, G. (1962). Biochem. J. 83, 41.
- Dixon, M. (1951). Manometric Methods. Cambridge : University Press.
- Emerson, M.R. (1954). Plant Physiol. 29, 418.
- Farkas, G.L. & Ledingham, G.A. (1959). Can. J. Microbiol. 5, 141.
- Fawcett, C.H., Spencer, D.M. & Wair, R.L. (1955). Ann. appl. Biol. 43, 553.
- Fodar, P.J. & Chari, A. (1949). Enzymylogia. 13, 258.
- Folley, S.J. & McNaught, Mary L. (1961). Milk: The Mammary Gland and Its Secretion. Chapter 12. Ed. by Kon, S.K. & Cowie, A.T. New York: Academic Press Inc.
- Forss, D.A., Dunstone, E.A. & Stark, W. (1960). J. Dairy Res. 27, 211.

- Foster, J.W. (1947). Bacteriol. Rev. 11, 167.
- Foster, J.W. (1949). Chemical Activities of Fungi. New York :
Academic Press Inc.
- Franke, W. & Heinen, W. (1958). Arch. Mikrobiol. 31, 50.
- Franke, W., Platzeck, A. & Eichhorn, G. (1961). Arch.
Mikrobiol. 40, 73.
- Franke, W., Platzeck, A. & Eichhorn, G. (1962). Arch.
Mikrobiol. 41, 154.
- Fredrickson, D.S. & Gordon, R.S. (1958). Phys. Rev. 38, 585.
- Garton, G.A. (1963). J. Lipid Res. 4, 237.
- Gehrig, R.F. & Knight, S.G. (1958). Nature, Lond., 182, 1237.
- Gehrig, R.F. & Knight, S.G. (1963). Appl. Microbiol. 11, 166.
- Gerson, T., Hawke, J.C., Shorland, F.B. & Melhuish, W.H.
(1960). Biochem. J. 74, 366.
- Girolami, R.L. & Knight, S.G. (1955). Appl. Microbiol.
3, 264.
- Glascock, R.F. (1958). Proc. Roy. Soc. (Lond.) Ser. B.
149, 402.
- Grafflin, A.L. & Green D.E. (1948). J. biol. Chem. 176, 95.

Graham, W.R., Jones, T.S.G. & Kay, H.D. (1936). Proc. Roy. Soc. (Lond.) Ser. B. 120, 330.

Green, D.E. (1954). Biol. Rev. 29, 330.

Green, D.E. (1963). Progress in the Chemistry of Fats and other Lipids. Chap. 3. Ed. by Holman, R.T., Lundberg, W.O. & Malkin. T. Oxford : Pergamon Press.

Haidle, C.W. & Knight, S.G. (1960). Biochim. Biophys. Acta. 39, 536.

Haller, A. & Lassieur, A. (1910). Compt. Rend. Acad. Sci. 150, 1013.

Hammer, B.W. & Bryant, H.W. (1939). Iowa State Coll. J. Sci. XI, 281.

Hansen, R.P. & Shorland, F.B. (1952). Biochem. J. 52, 207.

Hardwick, D.C., Linzell, J.L. & Mapham, J.B. (1963). Biochem. J. 88, 213.

Harper, W.J. (1953). J. Dairy Sci. 36, 808.

Harper, W.J. (1959). J. Dairy Sci. 42, 207.

Hawke, J.C. (1957). J. Dairy Res. 24, 366.

Henry, K.M. & Kon, S.K. (1942). Biochem. J. 36, 456.

Hilditch, T.P. (1956). The Chemical Constitution of Natural Fats 3rd. ed. revised. London : Chapman & Hall Ltd.

- Hiscox, E.R. (1941). J. Dairy Res. 12, 155.
- Holley, A.D. & Holley, R.W. (1952). Anal. Chem. 24, 216.
- Horiuchi, T., Horiuchi, S. & Mizuno, D. (1959). Nature, Lond., 183, 1529.
- Huelin, E.F. (1952). Austral. J. Sci. (Ser. B) 5, 328.
- Imamura, T. & Tsugo, T. (1953). Int. Dairy Congr. 4, 622.
- Irvine, O.R. Bullock, D.H. & Sproule, W.H. (1954). J. Dairy Sci. 37, 637.
- Jack, E.L. & Smith, L.M. (1956). J. Dairy Sci. 39, 1.
- Jackson, H.W. (1958). Perf. & Ess. Oil. Rec. 49, 256.
- Jackson, H.W. & Hussong, R.V. (1958). J. Dairy Sci. 41, 920.
- James, A.T., Peeters, G. & Lauryssens, M. (1956). Biochem. J. 64, 726.
- James, A.T., Lovelock, J.E. & Webb, J.P.W. (1959). Biochem. J. 73, 106.
- Jones, L.A., Holmes, J.C. & Seligman, R.B. (1956). Anal. Chem. 28, 191.

- Karrer, P. & Haab, F. (1948). *Helv. Chim. Acta.* 31, 795.
- Katz, Ira. & Chaikoff, I.L. (1955). *Biochim. Biophys. Acta.* 18, 87.
- Keeney, M. (1956). *J. Assoc. Off. Agri. Chem.* 39, 212.
- Keeney, M. (1957). *Anal. Chem.* 29, 1489.
- Keeney, M. & Day, E.A. (1957). *J. Dairy Sci.* 40, 874.
- Kirsch, D. (1935). *J. biol. Chem.* 108, 421.
- Klein, F. & de Jong, K. (1956). *Rec. trav. chim.* 75, 1285.
- Kluyver, A.J. & Perquin, L.H.C. (1933). *Biochem. Z.* 266, 68.
- Knight, S.G. (1963). Personal Communication.
- Knoop, F. (1904). *Beitr. chem. Physiol. Path.* 6, 150.
- Kosikowski, F.V. (1957). *Symp. Chem. Nat. Food Flavours.*
Page 133. Quartermaster Food Inst. Washington.
- Kosikowski, F.V. & Mocquot, G. (1958). *Advances in Cheese Technology.* F.A.O. Agricultural Studies No. 38.
- Kosikowski, F.V. (1961). *Proc. Flavour Chemistry Symposium.*
Campbell Soup Company.
- Kristoffersen, T. & Gould, I.A. (1959). *XVth Int. Dairy Congr.* 2, 920.

- Kristoffersen, T., Gould, I.A. & Harper, W.J. (1959).
Milk Prod. J. 50 (5), 14.
- Kristoffersen, T. & Cole, D.D. (1960). Milk Prod. J. 51 (7), 6.
- Kristoffersen, T. & Gould, I.A. (1960). J. Dairy Sci.
43, 1202.
- Kristoffersen, T. (1961). Proc. Flavour Chemistry
Symposium. Campbell Soup Company.
- Kumar, S., Lakshmanan, S. & Shaw, J.C. (1959). J. biol.
Chem. 234, 754.
- Lardy, H.A. (1951). Phosphorus Metabolism Vol. I. Baltimore,
Md : John Hopkins Press.
- Lauryssens, M., Verbeke, R., Peeters, G. & Reinards, M.
Thérèse. (1960). J. Dairy Res. 27, 151.
- Lawrence, R.C. (1962). Unpublished results.
- Lawrence, R.C. (1963). J. Dairy Res. 30, 161.
- Laxa, -. (1902). Arch. Hyg. 41, 127.
- Lehninger, A.L. (1945). J. biol. Chem. 161, 437.
- Levine, S. & Novak, M. (1949). J. Bact. 57, 93.
- Levinson, H.S. & Hyatt, M.T. (1963). Ann. N.Y. Acad.
Sci. 102, 773.

- Luick, J.R. (1961). J. Dairy Sci. 44, 652.
- Luick, J.R. & Lucas, J.M. (1962). Proc. Soc. Exp. Biol. Medicine. 110, 275.
- Lynen, F. (1955). Ann. Rev. Biochem. 24, 653.
- Lynen, F. (1961). Fed. Proc. 20, 941.
- Lynn, W.S., Steele, L.A. & Staple, E. (1956). Anal. Chem. 28, 132.
- Mabbitt, L.A. & Zielinska, M. (1956). XIV Int. Dairy Congr. II (2), 323.
- Mabbitt, L.A. (1961). J. Dairy Res. 28, 303.
- Magasanik, B. (1957). Ann. Rev. Microbiol. 11, 221.
- Magasanik, B. (1961). Cold Spring Harb. Symp. Quant. Biol. 26, 249.
- Mann, T. (1944). Biochem. J. 38, 339.
- McCarthy, R.D., Patton, S. & Evans, Laura (1960). J. Dairy Sci. 43, 1200.
- McCormick, N.G. & Halvorson, H.D. (1963). Ann. N.Y. Acad. Sci. (1963). 102, 763.
- McGugan, W.A. & Howsham, S.G. (1962). J. Dairy Sci. 45, 495.

- McGugan, W.A. (1963). *Can. Food Ind. Sept.* p.44.
- Metcalf, L.D. (1960). *Nature, Lond.*, 188, 142.
- Meyers, E. & Knight, S.G. (1958). *Appl. Microbiol.* 6, 174.
- Monty, K.J. (1958). *Anal. Chem.* 30, 1350.
- Morgan, M.E. & Anderson, E.O. (1956). *J. Dairy Sci.* 39, 253.
- Morgan, D.M. & Kingsbury, K.J. (1959). *The Analyst.* 84, 409.
- Morris, H.A. & Jezeski, J.J. (1953). *J. Dairy Sci.* 36, 1285.
- Morris, H.A., Jezeski, J.J., Combs, W.B. & Kuremoto, S.
(1963). *J. Dairy Sci.* 46, 1.
- Mukherjee, S. (1951). *Arch. Biochem. & Biophys.* 33, 364.
- Mulder, H. (1952). *Neth. Milk & Dairy J.* 6, 157.
- Munford, R. (1964). Personal communication.
- Nawar, W.W., Cancel, L.E. & Fagerson, I.S. (1962). *J. Dairy Sci.* 45, 1172.
- Nelson, F.E. & Babel, F.J. (1946). *Rep. Ia. Agric. Exp. Sta.*
Part I. p.210.
- Nelson, W.O. (1952). *J. Dairy Sci.* 35, 455.
- Nesbitt, J.M. (1953). *Dairy Sci. Abs.* 15, 821.

- Niedhardt, F.C. (1960). J. Bacteriol. 80, 536.
- Nieman, C. (1954). Bact. Rev. 18, 147.
- Olson, H.C. & Hammer, B.W. (1934). J. Dairy Sci. 17, 613.
- Oro, J.F., Guidry, C.L. & Zlatkis, A. (1959). Food Res. 24, 240.
- Parks, O.W., Keeney, M. & Schwartz, D.P. (1961). J. Dairy Sci. 44, 1940.
- Parks, O.W., Keeney, M., Katz, Ira & Schwartz, D.P. (1964). J. Lipid Res. 5, 232.
- Passman, J.M., Radin, N.S. & Cooper, J.A.D. (1956). Anal. Chem. 28, 484.
- Parmelee, C.E. & Nelson, F.E. (1949). J. Dairy Sci. 32, 1001.
- Patton, S. (1950). J. Dairy Sci. 33, 680.
- Patton, S. & Keeney, M. (1958). 41, 1288.
- Patton, S., Wong, N.P. & Forss, D.A. (1958). J. Dairy Sci. 41, 857.
- Patton, S. & Tharp, B.W. (1959). J. Dairy Sci. 42, 49.
- Patton, S. (1961). J. Dairy Sci. 44, 203.
- Patton, S. (1963). J. Dairy Sci. 46, 856.

- Patton, S. & McCarthy, R.D. (1963). J. Dairy Sci. 46, 916.
- Perry, K.D. & McGillivray, W.A. (1964). J. Dairy Res.
In Press.
- Peters, I.I. & Nelson, F.E. (1948). J. Bact. 55, 581.
- Peterson, M.H. & Johnson, M.J. (1949). J. Bact. 58, 701.
- Popják, G. & Beeckmans, M.L. (1950). Biochem. J. 46, 547.
- Popják, G., French, H.T. & Folley, S.J. (1951). Biochem. J.
48, 411.
- Popják, G., French, H.T., Hunter, G.D. & Martin, A.J.P.
(1951). Biochem. J. 48, 612.
- Popják, G. (1952). Biochem. Soc. Symp. No.9, 37.
- Proks, J., Dolezalek, J. & Pech, Z. (1959) XVth Int. Dairy
Congr. 2, 729.
- Rapkin, E. (1962). Packard Technical Bulletin No. 7.
- Raveux, R. (1948). C.R. Acad. Sci. (Paris) 231, 729.
- Reid, E.E. & Ruhoff, J.R. (1944). Organic Synthesis Vol. II
Ed. Blatt, H.A. New York : John Wiley and Sons.
- Rigler, N.E. & Greathouse, G.A. (1940). Am. J. Bot. 27, 701.
- Riis, P.M., Luick, J.R. & Kleiber, M. (1960). Am. J. Physiol.
198, 45.

- Robertson, S. (1957). *Physiol. Revs.* 37, 133.
- Robertson, P.S. (1961). Ph.D Thesis. University of Reading.
- Robertson, P.S. & Perry, K.D. (1961). *J. Dairy Res.* 28, 245.
- Robertson, P.S. (1964). Private Communication.
- Rolinson, G.N. (1954). *J. Appl. Bact.* 17, 190.
- Roper, S.R. & Ma, T. (1957). *Microchem. J.* 1, 245.
- Scarpellino, R.J. (1961). *Diss. Abstr.* 22, 421.
- Schwartz, D.P. & Parks, O.W. (1961). *Anal. Chem.* 33, 1396.
- Schwartz, D.P., Parks, O.W. & Keeney, M. (1962). *Anal. Chem.* 34, 669.
- Schwartz, D.P., Haller, H.S. & Keeney, M. (1963). *Anal. Chem.* 35, 2191.
- Scott, J.K. (1955). *J. Dairy Res.* 22, 302.
- Seitz, E.W., Sandine, W.E., Elliker, P.R. & Day, E.A. (1963). *Can. J. Microbiol.* 9, 431.
- Shaw, J.C. & Knodt, C.B. (1941). *J. biol. Chem.* 138, 287.
- Shaw, J.C., Powell, R.C.J. & Knodt, C.B. (1942). *J. Dairy Sci.* 25, 909.

- Sherman, J.R. (1963). Anal. Biochem. 5, 548.
- Sherwood, I.R. (1939). J. Dairy Res. 10, 326.
- Shipe, W.F. (1951). J. biol. Chem. 30, 165.
- Shorland, F.B. (1952). J. appl. Chem. 2, 438.
- Silverman, M. & Kosikowski, F.V. (1953). J. Dairy Sci. 36,
574.
- Simon, E.W. (1950). Nature, Lond., 166, 343.
- Simon, E.W. & Beevers, H. (1951). Science. 114, 124.
- Singh, V.N. & Kumar, S. (1963). Biochim. Biophys. Acta.
70, 711.
- Sjöström, G. & Malm, B. (1952). Sv. Mejeritidn. 44, 81.
- Sjöström, G. (1958). Sv. Mejeritidn. 50, 655.
- Sjöström, G. (1959). Milk & Dairy Res. Alnarp, Sweden.
Report No. 58.
- Stadhouders, J. (1956). Diss. Wageningen.
- Stadhouders, J. & Mulder, H. (1957). Neth. Milk Dairy J. 11,
164.
- Stadhouders, J., De Vries, E. & Mulder, H. (1959). Int.
Dairy Congr. 2, 709.
- Stadtman, E.R. & Barker, H.A. (1949). J. biol. Chem. 180, 1108.

- Starkle, M. (1924). *Biochem. Z.* 151, 371.
- Stokoe, W.N. (1928). *Biochem. J.* 22, 80.
- Stumpf, P.K. & Bradbeer, C. (1959). *Ann. Rev. Plant Physiol.* 10, 197.
- Sundt, E. & Winter, M. (1957). *Anal. Chem.* 29, 851.
- Sussman, A.S., Distler, J.R. & Krakow, J. (1956). *Plant Physiol.* 31, 126.
- Suzuki, S.K., Hastings, E.G. & Hart, E.B. (1910). *Res. Bull. Wisc. Agric. Sta. No. 11.*
- Thaler, H. & Geist, G. (1939). *Biochem. Z.* 302, 121.
- Thaler, H. & Eisenlohr, W. (1941). *Biochem. Z.* 308, 88.
- Thaler, H. & Stahlin, Ilse. (1949). *Biochem. Z.* 320, 84.
- Thibodeau, R. & Macy, H. (1942). *Minn. Sta. Tech. Bull.* 152.
- Thornton, R.H. (1963). *N.Z. J. Agric. Res.* 6, 318.
- Torriani, A. (1960). *Biochim. & Biophys. Acta.* 38, 460.
- Turner, J.S. & Hanly. VI (1947). *Nature, Lond.*, 160, 296.
- Urbach, Gerda. (1963). *J. Chrom.* 12, 196.

- Vagelos, P.R. & Alberts, A.W. (1960). J. biol. Chem. 235, 2786.
- Van der Ven, B., Haverkamp Begmann, P. & Schogt, J.C.M. (1963).
J. Lipid Res. 4, 91.
- Vinze, V.L. & Ghosh, D. (1961). Hind. Antibiotics Bull. 4,
No. 1.
- Vinze, V.L. & Ghosh, D. (1962). Hind. Antibiotics Bull. 4,
No. 3.
- Walker, J.R.L. & Harvey, R.J. (1959). J. Dairy Res. 26, 265.
- Walker, J.R.L. (1961). J. Dairy Res. 28, 1.
- Wakil, S.J. (1961). J. Lipid Res. 2, 1.
- Wakil, S.J. (1962). Ann. Rev. Biochem. 31, 369.
- Webley D.M. & de Kock, P.C. (1952). Biochem. J. 51, 371.
- Weinstein, S.S. & Wynne, A.M. (1936). J. biol. Chem. 112, 649.
- Wilcox, J.C., Nelson, W.O. & Wood, W.A. (1955). J. Dairy
Sci. 38, 775.
- Windlan, H. (1955). Ph.D. Thesis, Cornell University.
- Witting, L.A. & Batzer, O.F. (1957). Food Res. 22, 237.
- Wong, N.P., Patton, S. & Forss, D.A. (1958). J. Dairy
Sci. 41, 1699.

Wood, W.A. (1961). The Bacteria Vol. II. Chapt. 2.

Ed: Gunslaus, I.C. & Stanier, R.Y. New York : Academic Press.

Wyss, O., Ludwig, B.J. & Joiner, R.R. (1945). Arch.

Biochem. 7, 415.

ABSTRACT

The similarity of the pattern of methyl ketones obtained from the steam distillates at atmospheric pressure of cheeses made under controlled aseptic conditions, despite the wide differences in bacterial flora, led to the suspicion that the methyl ketones were being formed as artifacts. This was confirmed by steam distilling cheeses from 1 day to 13 months old when the qualitative patterns and quantitative amounts differed little with the age of the cheese. Evidence was produced to show that the greater part of these methyl ketones must be formed during the heat treatment of milk fat. The maximum quantities of methyl ketones obtainable from cheese and from milk fat, determined by exhaustive steam distillation at atmospheric pressure, averaged from 14 p.p.m. for 2-undecanone to 46 p.p.m. for 2-pentadecanone. Some artifact formation of methyl ketones also occurred, although to a greatly reduced extent, when dairy products containing milk fat were steam distilled under reduced pressure at 40°. As methyl ketones in low concentrations could be extracted from mature cheese at room temperature by solvents or by flushing cheese suspension with nitrogen, milk fat appears to contain precursors which break down to methyl ketones slowly during cheese ripening, this breakdown being accelerated at higher temperatures.

Two possible modes of formation of the methyl ketones with an odd number of carbon atoms, found in limiting quantity in the steam distillates of Cheddar cheese, were considered:-

- (a) From precursors, probably β -keto acids, bound in milk fat.
- (b) The β -oxidation of free fatty acids, formed by the lipolysis of milk triglycerides, and subsequent decarboxylation of the β -keto acids formed.

The use of radioactive milk fat from a lactating cow which had been injected intravenously with carboxy- ^{14}C acetate allowed a direct comparison to be made between the labelling patterns of fatty acids and the corresponding methyl ketones from the same milk source. The similarity of the labelling patterns suggests that the C_6 to C_{16} β -keto acids and the corresponding fatty acids have a common precursor (or that one is the precursor of the other) and are together incorporated into the triglycerides. Only butyric acid of the C_4 to C_{16} fatty acids in all 3 milkings had a higher specific activity than the corresponding methyl ketone. This suggests that the acetone found in steam distillates of milk fat is formed from a compound (probably D- β -hydroxybutyrate), derived almost entirely from a precursor other than acetate.

The finding that the saturated C_{18} acid in all 3 samples of radioactive milk fat had an extremely low activity was in agreement with the fact that no C_{17} methyl ketone was detected in any of the numerous steam distillates from milk fat or cheese. This supports the generally accepted view that, in the biosynthesis of milk fat, the fatty acids up to C_{16} acid are synthesised from an acetate pool, whereas C_{18} acids and above are obtained from the blood triglycerides.

The possibility that methyl ketones were being formed in Cheddar cheese from the β -oxidation of free fatty acids, as well as from a slow breakdown of bound β -keto acids in milk fat, was shown to be improbable. Triglycerides of acids (undecanoic, nonanoic, and heptanoic), which occur normally only in traces in milk fat, were synthesised and incorporated in Cheddar cheeses. On steam distillation of these cheeses when mature, no methyl ketones corresponding to the acids in the added triglycerides were obtained, although the normal range of methyl ketones with an odd number of carbon atoms was found in the distillates.

A detailed study of the metabolism of fatty acids and synthetic triglycerides by spores and mycelium of Penicillium roqueforti was undertaken, this fungus being chosen as a general representative of lipolytic organisms that might be of importance in producing Cheddar flavour. The effect of the growth medium, pH of solution, concentration of acid and inorganic ions on both oxygen uptake and methyl ketone formation was determined.

The rate of methyl ketone formation suggested the synthesis of adaptive β -keto acid decarboxylases after a lag period of 1 to 2 hours. An hypothesis based upon the possible toxicity of the C_6 to C_{12} β -keto acids can explain a number of the experimental results:

- (1) Only one methyl ketone was formed
- (2) The most toxic acids were those which gave the least amount of methyl ketone

(3) Concentrations of the C₈ to C₁₂ fatty acids that markedly inhibited the respiration of mycelium were nevertheless oxidised to considerable quantities of the corresponding methyl ketone.

The relationship between the toxic action of fatty acids and chain length was found to be dependent upon pH. The chain length of the most toxic acid increased with pH, being C₁₀ acid at pH 2.5 and C₁₂ at pH's 5.2 and 6.0. At pH 6.8 none of the acids from C₄ to C₁₈ inhibited oxygen uptake but C₁₄ acid was the most toxic acid at pH 8.0.

There appeared to be no sharp dividing line between the metabolic activity, with respect to fatty acids, of spores and mycelium. Mycelium oxidised fatty acids rapidly giving varying amounts of methyl ketone but considerably more CO₂ than spores. In general spores formed higher amounts of methyl ketones than mycelium but showed also a slight but definite ability to form CO₂ from octanoic acid.

Evidence for a β -oxidation mechanism in the fungal metabolism of fatty acids was obtained by the use of 1-C¹⁴ and 2-C¹⁴ octanoic acids. A relatively slow movement of intermediates through the T.C.A. cycle was also indicated.

High concentrations of triglycerides were oxidised slowly by spores to methyl ketones when equivalent concentrations of the free acid (up to 66 μ moles/ml) inhibited methyl ketone formation. It seems probable that the very slow rate of formation of methyl ketones is due to the inhibitory effect of the ketones themselves on the lipases.

Methyl carbinols were not detected in significant amounts as products of the metabolism of methyl ketones,

evidence being obtained on the contrary that the carbinols were possibly precursors of the ketones during the oxidation of fatty acids by spores.

Cell free extracts obtained from mycelium were able to oxidise low concentrations of octanoic acid (0.5 μ moles/ml or less) after a lag of up to 3 hours. The supplementation of the extracts with several coenzymes, known to be associated with fatty acid oxidation, or with T.C.A. cycle intermediates were unable, however, to decrease the lag before oxidation started. No methyl ketones were detected after the oxidation of fatty acids by cell-free extracts but were formed when cell debris from the Hughes Press was used. This suggests that the β -keto acid decarboxylases were tightly bound to the cell walls.