THE EFFECT OF BIOTIN ON ACETATE UTILIZATION AND LIPIDE SYNTHESIS BY MICROORGANISMS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science
in
The Department of Agricultural Chemistry and Biochemistry

by
James Thomas Jackson
B. S., Louisiana State University, 1949
August, 1952

ACKNOWLEDGMENT

The writer wishes to express his sincere gratitude to Dr. John F. Christman, under whose direction this work was performed.

Grateful acknowledgment is also made to Dr. Virginia R. Williams for her guidance in the completion of this work and in the preparation of this manuscript.

The writer also wishes to express his appreciation to the National Institutes of Health, United States Public Health Service, for support as a Research Assistant in the course of this work.

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ABSTRACT

Because relatively little is known concerning the intermediary metabolism of lipides, this field offers one of the major challenges to present day researchers. Several mechanisms have been postulated for the biological oxidation of fatty acids and of these the beta-oxidation theory has in recent years received support from tracer work. In as far as biosynthesis of fatty acids is concerned, while there is very little evidence available concerning the mechanism, it is known that these acids can be synthesized from a number of short chain compounds such as acetic acid, and that this synthesis occurs by condensation of two carbon units. On the basis of such evidence as this, it is conceivable, as suggested by several workers, that the mechanism of synthesis might involve the reverse of oxidation.

In the light of the stimulatory effect that certain lipides are known to exert on a variety of microorganisms and the fact that in a number of cases this stimulation has been correlated with biotin activity, the effect of biotin on the utilization of acetate for fatty acid synthesis has been studied. Radioactive sodium acetate was incubated in the presence of cells of high and low biotin content and cells of low biotin content with biotin added to the incubation mixture. The long chain fatty acids were isolated and their radioactivity determined.

A significant synthesis of radioactive fatty acids by cells of Lactobacillus casei has been demonstrated; however, any conclusions concerning the effect of biotin on this synthesis will have to await resolution of the fatty acid mixture, a separate phase of the general problem. In agreement with many investigators it has been demonstrated that of the utilized acetate, by far the largest amount was concentrated in the non-saponifiable, ether soluble fraction. In addition it has been shown that for cells grown on a complete medium very little or no activity was recovered in the fatty acid fraction in contrast to cells grown on a synthetic medium for which significant amounts of activity were recovered.

INTRODUCTION AND HISTORICAL.

There is now available a considerable amount of evidence concerning lipide stimulation of the growth of various microorganisms and it is known that certain of the unsaturated long chain fatty acids can replace biotin in the metabolism of several of these microorganisms. There is, however, very little evidence of a direct nature available concerning the mode of action of lipide stimulation and biotin replacement. Since a biotin-lipide interrelationship is known, several possible mechanisms present themselves: (1) that certain lipides are concerned in the synthesis of biotin, (2) that biotin is concerned in the synthesis of these lipides or (3) that both biotin and these lipides have a function in common and the one can be dispensed with in the presence of the other. All of the evidence now available deals either with the first or last of these possibilities. Evidence of a direct nature concerning the second possible mechanism is very desirable since it would tend to confirm or disprove one or more of these suggested mechanisms.

In view of the fact that acetate can be utilised by certain microorganisms and tissues for the synthesis of fatty acids it was considered possible to devise a working system with which to study the effects of biotin on the utilisation of acetate for the synthesis of long chain fatty acids. Thus, it was proposed to incubate cells of

high and low biotin content, and cells of low biotin content containing added biotin, in the presence of radioactive carbon C-14 labeled sodium acetate; to isolate the total long chain fatty acids from the incubation mixture; subsequently to resolve this mixture into its components; and finally to determine whether or not biotin had any effect on the radioactivity of one or more of the unsaturated fatty acids, particularly oleic acid. Once a working system of this sort was established it would further be possible to study the effects of a number of other vitamins on the synthesis of fatty acids and in the long run to contribute some knowledge to the mechanism of fatty acid synthesis.

I. Lipide Stimulation in Bacterial Metabolism.

The bactericidal and hemolytic activity of the higher unsaturated fatty acids or their sodium salts was known at least as early as 1907 from the work of Noguchi(1). In 1911 Lamar(2) showed that the hemolytic activity of these acids was directly related to their degree of unsaturation. This worker also demonstrated that normal goat serum inhibits the destructive action of the higher fatty acids. Although the reasons for the toxic nature of these fatty acids are not clearly understood, it is generally agreed, as noted above(2) and elsewhere(3, 4, 5, 6), that toxicity is observed only with the unesterfied acids.

Probably the first recognized inclusion of oleic acid as a growth promoting agent in bacteriological media was carried out in 1909 by Fleming(7) for the cultivation of the acne bacillus. A few

years later, in the development of a selective medium for Hemophilus influenzae, Avery(8) made use of the bactericidal activity of the soaps of the higher unsaturated fatty acids. He found that the addition of sodium oleate to media prevented the growth of certain gram-positive organisms while the growth of H. influenzae was enhanced by its presence. On this oleate-hemoglobin medium the gram-negative cocci of the Neisseria catarrhalis group, staphylococci, and occasionally diphtheroid bacilli grew.

In 1933 Loebel and coworkers(9), while studying the influence of various materials upon the respiration and growth of the human tubercle bacillus, demonstrated a very marked increase in oxygen uptake in the presence of as little as 0.1 per cent soap. Lecithin, milk and serum fat also showed good stimulation. Credit, however, is usually given to Cohen, Snyder and Mueller(10) for first demonstrating the essential nature of a particular fatty acid. These workers isolated from serum, milk, and commercial casein two growth factors for certain strains of diphtheroid bacilli. Although neither factor was effective in the absence of the other, one was shown to be oleic acid. The stimulation phenomenon by oleic acid was subject to a relatively sharp optimum concentration, lower levels than 1 mg of oleic acid per tube being insufficient and higher levels being inhibitory.

A short time later, the fungus Lipomyces ovale was reported by Benham(11) to grow in the presence of inorganic salts, glucose and oleic acid. Similarly, Feeney and coworkers(12, 13)

have shown that oleic acid functions as a growth factor for Clostridium tetani.

Hutner(3) found that soap was stimulatory to Erysipelothrix rhusiopathiae. Since high levels of soap proved toxic, substitutes for soap were investigated. One such material, saponin, although inactive by itself, served to detoxify the soap, which contained the active material. Oleic acid was found to be more effective than soap and permitted full growth only in the presence of saponin. Oleic acid without saponin was sharply inhibitory above the 0.002 per cent level, however, in the presence of saponin high concentrations of oleate were non-toxic.

In a study of the substances present in cereals and other biological materials, which interfere with the determination of riboflavin by the microbiological method, Streng and Carpenter(14) have shown that the interference is probably due to small amounts of free fatty acids. Similarly, Bauerfeind, Cotier and Boruff(15) have shown that certain fatty acids and other lipide materials have a stimulatory effect on the growth of Lactobacillus casei in the microbiological assays for riboflavin and pantothenic acid when suboptimum amounts of either of these vitamins is present in the assay medium.

Some years later Kodicek and Worden(4) found that linolenic acid, linoleic acid and to a lesser extent oleic acid, exert an inhibitory effect on the growth and acid production of Lactobacillus helveticus and other gram-positive bacteria. The methyl esters of these fatty acids

exerted no inhibitory action. The inhibitory effect of the fatty acids also could be overcome by addition of a number of compounds, such as lecithin, cholesterol, calciferol, lumisterol, and alpha-tocopherol.

Dubos(5) has found that certain complex lipides exert a remarkable stimulatory effect on the multiplication of mycobacteria. Particularly striking results were obtained with (a) phosphatide fractions prepared from egg yolk, cattle brain, human erythrocytes, and soyabeans and (b) synthetic non-ionic surface active agents consisting of esters of long chain fatty acids and of polyhydric alcohols. Later these same substances were found to enhance the growth of an unidentified Micrococcus(6). Dubos(5, 6) also found that the previously noted toxicity(1, 2, 8) of the free fatty acids could be overcome by using esters such as methyl oleate, triethanolamine oleate and phosphatides or by adding to the medium native serum albumin. When rendered non-toxic, a number of long chain fatty acids were found able to enhance growth of certain bacteria.

A few years ago Hutchings and Boggiano(16) showed that sodium oleate is necessary for maximum growth of several strains of lactic acid bacteria, the amount varying with each organism and increasing amounts becoming toxic towards Lactobacillus plantarum and Lactobacillus leichmannii. Even more recently Kitay and Snell(17) in a survey of the nutritive requirements of twenty-eight cultures of lactic acid bacteria previously reported not able to grow in media of known composition, found that all but two required oleic acid or other

unsaturated fatty acids for growth.

II. The Biotin-Lipide Interrelationship.

In the course of the microbiological assay of rice polish for biotin, Williams and Fieger(18) showed a disparity in the actual and apparent biotin content of the rice polish. These workers were able to demonstrate(19) that L. casei, the organism used in the assay, could be maintained for a number of months on an essentially biotin-free medium. In this work and a series of well-defined experiments these workers have established the biotin-oleic acid interrelationship and have contributed a major part of the known evidence concerning the mechanism involved.

In view of the previously noted "detoxification" of the fatty acids by esterification, a number of synthetic detergents were examined for activity (Williams and Fieger)(20). Nearly all of the twenty-four detergents examined proved stimulatory even at high concentration. Non-ionic oleates were, in general, the most stimulatory of the detergents tested. Later, several non-fatty acid surface-active agents were examined by Williams and Fieger(21), but none was found which would stimulate the growth of L. casei in the absence of biotin.

Since lipide stimulation of growth occured in the absence of any detectable amount of biotin(20), in contrast to lipide stimulation in media low but not deficient in riboflavin or pantothenic acid(14, 15), it was concluded by Williams and Fieger that biotin is not a component of an enzyme system as are the latter two vitamins. Such evidence as

this led these workers to suggest that the stimulation phenomenon is due to the surface-active nature of the fatty acids. Further evidence in favor of such a hypothesis has been offered by Williams and Williams(22), who by means of electrophoretic and polarographic studies have shown biotin to exhibit a surface active nature.

It is thought by several workers that the previously mentioned explanation of the mode of action of oleic acid, based on its surface activity, is not substantiated by sufficient evidence. In this connection Axelrod and coworkers(23) have offered the observation that numerous analogues of biotin are completely devoid of biotin activity, although on a structural basis they would be expected to be even more surface-active than biotin. Similarly, Potter and Elvehjem(24) feel that the data of Williams and Pieger are not incompatible with a nutritional role for biotin and oleic acid, unless it is shown that the detergents in their experiments are active in the absence of oleic acid or other fatty acids nutritionally important to the organism. Potter and Elvehjem base their objection on the observation by Williams, Broquist and Snell(25), that inactive detergents are active in the presence of oleic acid for some lactobacilli.

In view of such objections as these and in analogy with the involvement of biotin in aspartic acid synthesis as demonstrated by Lichstein and Umbreit(26) and Lichstein and Christman(27), Williams and coworkers(25) and Potter and Elvehjem(24) have suggested that biotin is involved in the synthesis of oleic acid. Likewise, it has been

shown recently by Broquist and Snell(28) and Andrews and Williams(29) that a synthesis of biotin occurs in cells of certain microorganisms grown on a medium containing oleic acid as a substitute for biotin. However, this evidence does not necessarily imply that oleic acid is involved in biotin synthesis.

Confirmation of the biotin-lipide interrelationship has been provided by Carlson and coworkers(30), who have found that fatty acids in the form of Tweens (Tween 80 being the polyoxyethylene derivative of sorbitan monooleate) can substitute for biotin in the metabolism of several strains of Leuconostoc. Similarly, Shull, Thoma, and Peterson(31) have shown that in addition to oleic and linoleic acids, found by other workers to be active in replacing biotin for lactic acid bacteria, vaccenic and ricinoleic acids are also active for Clostridium sporogenes.

Trager(32) recently described the isolation of a fat soluble fraction (FSF) from blood and plasma in the presence of which biotin was non-essential for growth of L. casei. Later(33) it was found that, while both oleic acid and FSF can replace biotin, the former material was active over a narrower range of pH than the latter, presumably because of its greater toxicity. It is now thought that FSF is an esterified form of oleic acid(25).

Axelrod and coworkers(23, 34) and Broquist and Snell(28) have shown that certain saturated fatty acids are capable of decreasing the requirement for unsaturated fatty acids under the conditions of their

experiments.

Whitehill and coworkers(35) have recently isolated a strain of Lactobacillus which does not grow in the absence of sodium oleate when all of the vitamins - including riboflavin, pantothenic acid and biotin - are present in a 5-fold excess of what is ordinarily considered sufficient for lactobacilli. This organism does not require acetate, and cannot grow in the absence of oleate, even in the presence of high levels of sodium acetate.

A growth stimulating effect of oleic and aspartic acids has been noted by Hodson(36) for the cholineless mutant of Neurospora crassa. Under the conditions used, however, these acids could not completely replace biotin for the growth of this organism. Oleic acid and Tween 80 alone or in combination with aspartic acid gave some growth response in the absence of biotin and a slight stimulatory response in the presence of biotin.

Lactobacillus arabinosus, L. casei, and Streptococcus faecalis have been found by Broquist and Snell(28) to require increased amounts of biotin for growth in the absence of aspartic acid. However, Lactobacillus fermenti and Clostridium butyricum require the same amount of biotin in the absence or presence of aspartic acid. For the first of these organisms, aspartic acid and unsaturated fatty acids are required to permit growth in the complete absence of biotin; for the latter two organisms, unsaturated fatty acids alone permit such growth. It was concluded that in contrast to the involvement of biotin in aspartic

acid synthesis(26), biotin is probably not involved in its synthesis by these latter two organisms.

It would appear from this discussion of the biotin-lipids interrelationship that further study is required before any definite conclusions can be drawn concerning the mechanism of the observed phenomenon.

III. Utilization of Acetate for Fatty Acid Synthesis.

With the advent of heavy and radioactive isotopes as metabolic tracers, a great number of articles have been published concerning the metabolism of fatty acids. Among the first of these works were those of Schoenheimer and Rittenberg(37-42) using deuterium as a tracer. The results of these workers gave new and supporting evidence for the beta-oxidation theory for the metabolic degradation of fatty acids as first demonstrated by Knoop. It has been known for some time that fats can readily be synthesized from carbohydrate. The studies of Schoenheimer and Rittenberg(37-42) have demonstrated that there is a rapid and continuous conversion of carbohydrate to fat under normal dietary conditions.

Using acetic acid, a likely intermediary in fat synthesis from carbohydrate, Rittenberg and Bloch(43) obtained evidence that fatty acids are synthesized by condensations of acetic acid, or of a compound into which acetic acid can readily be converted. These workers fed acetic acid labeled by deuterium in the methyl group and

C-13 in the carboxyl group to mice and rats and subsequently demonstrated the presence of both C-13 and deuterium in the total fatty acids. From these data they concluded that both the carbon atoms of acetic acid are used in the synthesis of some components of the total fatty acid mixture.

Barker, Kamen and Bornstein(44), using cells of Clostridium kluyveri, were able to show that acetic acid labeled in the carboxyl group with C-14 gave rise to butyric acid labeled almost equally in the carboxyl and beta-positions. The alpha and gamma positions were inactive. Similarly, caproic acid had one third of its C-14 in the carboxyl group. The beta and delta positions were not examined for activity. These workers also showed that when C. kluyveri is grown with ordinary ethanol and synthetic carboxy-labeled butyric acid, C-14 was found in caproic acid but not in acetic acid. The active caproic acid so formed contained almost no activity in its carboxyl group. This would seem to indicate, as Barker and his associates pointed out, that caproic acid formation involves a condensation of the carboxyl group of butyric acid or some related C_4 compound with the methyl group of acetic acid.

Guirard, Snell and Williams(45) have offered confirmatory evidence of a non-tracer character for the above noted synthesis of fatty acids from acetate. They have shown that representatives of related types of compounds - fatty acids, keto acids, sterols, bile acids, sex hormones, saponins, heart poisons, resin acids, fat soluble vita-

mins, terpenes and carotenoids - can replace acetate in varying degrees in its growth-stimulating capacity for a number of different species of lactic acid bacteria. They concluded that acetate may be utilized by these organisms for the synthesis of lipoid materials.

Brady and Gurin(46) have shown that rat liver slices are capable of synthesizing long chain fatty acids from acetic, pyruvic, hexanoic, and octanoic acids. The results of these workers suggest that the synthesis of long chain fatty acids from short chain acids probably occurs, to a large degree by fragmentation of these acids to 2-carbon units which are subsequently recombined to form long chain fatty acids.

Although very little is known concerning the mechanism of synthesis of the long chain fatty acids, on the basis of the evidence now available, it seems certain that long chain fatty acids are produced from acetate by a variety of tissues, cells, and cell-free enzyme preparations and that chain elongation occurs by 2-carbon atom fragments.

On the basis of the preceding discussion the phenomenon of lipide stimulation now seems well established as does the biotin-lipide interrelationship, thus a considerable interest has been aroused in determining the mechanism by which certain lipides can replace biotin in the metabolism of various microorganisms. Concerning the different possible mechanisms of interrelation there is available very little data of a direct nature; however, a considerable amount of

evidence points to a mechanism based upon the surface-active nature of these substances and recently it has been shown that there is a synthesis of biotin in cells grown on Nopalcol 6-0. A third possible mechanism, namely that biotin is involved in the synthesis of oleic acid, though postulated by several workers, is as yet neither confirmed nor discredited by experimental evidence.

In view of the known synthesis of fatty acids from radioactive acetate, a method appears to be available for studying the effect of biotin upon acetate utilization for fatty acid synthesis. Perhaps the most serious handicap to such a proposed experiment is the absence of a good method for separating micro quantities of the total long chain fatty acid mixture into its components.

EXPERIMENTAL METHODS

The experiments were divided, in general, into four phases, which are: (1) growth and harvestation of cells of high and low biotin content; (2) incubation of these cells in the presence of acetate; (3) isolation of total fatty acids from the incubation mixture; and (4) determination of their radioactivity.

Growth and harvestation of cells:

Stock cultures of Lactobacillus casei ATCC 7469, obtained from the American Type Culture Collection, Washington, D.C., were carried as stab cultures in yeast extract-glucose agar. Inocula for use in the tests were prepared by inoculating from the stock cultures into a tube of sterile yeast extract-sodium acetate-glucose broth.

The synthetic high and low biotin content media for inoculation were prepared as described by Shull, Hutchings, and Peterson (47) and revised in 1948, for the microbiological assay of biotin. Two separate 2 liter flasks containing one liter of medium each were prepared. To one liter of the medium was added 0.8 microgram of biotin and to the other flask was added 80 milligrams of Nopalcol 6-0, a synthetic non-ionic oleate obtained from the National Oil Products Company (Nopco), Harrison, New Jersey. The medium was next autoclaved for 15 minutes at 15 pounds pressure and allowed to cool. Each

flask was inoculated with one milliliter of a 24 hour culture of L. casei in yeast extract-sodium acetate broth.

The culture was incubated at 37°C for 24 hours, harvested by centrifugation, washed once in distilled water and recentrifuged. The cells were then taken up in a small amount of water and used as such for the inoculation in the second phase.

Incubation of the cells in the presence of acetate:

A series of six tubes was set up containing the ingredients indicated in Table I. The tubes were then placed in a 37°C water bath and allowed to incubate for two hours. At the end of the incubation period the cells were destroyed by heating for 5 minutes in boiling water.

Several experiments were made, as described above, using non-radioactive acetate in order to determine the optimum conditions and establish the procedure. Later an experiment was carried out, under the same conditions (Table I), using instead of sodium acetate, radioactive carboxy C-14 labeled sodium acetate, obtained from Tracerlab Inc., Boston, Massachusetts. The radioactive material was added to the working tubes (B, C, E, and F) to the extent of 0.01 millicurie. A second experiment using radioactive acetate was set up as indicated in Table II.

Isolation of total fatty acids:

The method used for the quantitative extraction for total fatty

TABLE I

Contents of Incubation Mixtures

Tube No.	Cells in ml	pH 7.0 phosphate buffer in ml	Na_2CO_3** in ml	Biotin in ug	Acetate in mg
					Active / Non-active
A	5*	5	1	-	-
B	5*	5	1	-	5
C	5*	5	1	-	25
D	5§	5	1	-	-
E	5§	5	1	-	25
F	5§	5	1	1	25

* Cells grown on a synthetic medium containing added biotin.
§ Cells grown on a synthetic medium containing Nopalcol 6-0 (a synthetic non-ionic oleate).
** Sodium carbonate was included since it has been found by Block(46) and by Brady and Gurin(46) that fatty acid synthesis is enhanced by carbonate buffer.

All tubes were made to a total volume of 17 ml.

TABLE II

Contents of Incubation Mixtures

Tube No.	Cells in ml	pH 7.0 phosphate buffer in ml	Na_2CO_3** in ml	Biotin in ug	Acetate in mg	
					Active	Non-active
A	5*	5	1	-	-	8
B	5*	5	1	-	8	-
C	5ϕ	5	1	-	-	8
D	5ϕ	5	1	-	8	-
E	5ϕ	5	1	1	8	-

* Cells grown on a synthetic medium containing added biotin.

ϕ Cells grown on a synthetic medium containing Nopalcol 6-0 (a synthetic non-ionic oleate).

** See Table I.

All tubes were made to a total volume of 17 ml.

acids was that of Kaplan and Chaikoff(49) and was as follows:

To the 17 ml of incubation material was added enough ethyl alcohol to give a volume of 100 ml following which 10 gm of sodium hydroxide pellets was added (sodium hydroxide was used instead of potassium hydroxide because of the inherent radioactivity of potassium). The mixture was refluxed for four hours and then approximately 20 ml of the ethyl alcohol was evaporated. The contents of the flasks were transferred to 500 ml Squibb separatory funnels, sufficient water being used in the transfer to decrease the concentration of alcohol to 25 per cent.

The saponified material was extracted with four portions of ethyl ether and any soaps removed from the ether by three extractions with 15 ml portions of water, these water washings being added back to the saponifiable fraction.

The saponifiable fraction was next transferred to 250 ml centrifuge bottles and the saponified fatty acids were subjected to precipitation by the copper-lime procedure of Lehninger and Smith(50) as used by Brady and Gurin(46). According to these latter workers, those fatty acids whose chain lengths are longer than 10 carbon atoms are found in the copper-lime precipitate, whereas all those of shorter chain length remain in the supernatant. This procedure effectively separates any unused acetate and permits the isolation of those long chain fatty acids in which this work is concerned.

The precipitate was subsequently washed three times with

water, acidified, reprecipitated with concentrated sodium hydroxide, and washed three additional times. The copper-lime precipitate was then treated with 6 N hydrochloric acid and the long chain fatty acids extracted with petroleum ether (Skelly solvent "B", B.P. approximately 60°C).

The petroleum ether extract was washed with 0.1 N hydrochloric acid and evaporated to dryness. In preliminary experiments the recovered fatty acids were transferred to tared beakers and weighed. In the later experiments the fatty acids were taken up in petroleum ether and prepared for counting as described below.

Determination of radioactivity:

The petroleum ether fractions containing the fatty acids were concentrated to approximately 3 ml volume and transferred with washings to a 10 ml volumetric flask which was subsequently made up to volume with petroleum ether. Aliquots of these samples were placed on 1 inch watch glasses and the solvent evaporated under an infra-red lamp.

The watch glass containing the sample to be counted was introduced into the counting chamber of a Nuclear model D46 "Q-gas" counter attached to a Nuclear model 161 scaling unit. The counter was then "out-gassed" for 10 minutes, following which a 10 minute count was made. All counts are expressed as counts per minute above background which, in the preliminary experiments, was taken as the counts per minute of tubes A and D, and in the later experiments, as the counts

per minute with a clean watch glass in the counter.

The experiments to be described here are divided into two main groups: (1) the preliminary experiments involving the use of ordinary sodium acetate, and (2) the main experiments, in which radioactive acetate was used.

Experiments involving the use of ordinary sodium acetate.

The initial experiments using non-radioactive acetate, summarized in Table III, were designed to determine the optimum conditions and to establish procedure for use in the tracer work. Since, as can be seen from Table III, the results showed no simple relationship between the various samples and the weight of the fatty acids produced, it was decided to continue the experiments with radioactive acetate.

Experiment involving radioactive sodium acetate:

Table IV shows the results of an experiment which was carried out exactly as Experiment 3 (Table III), except that the working tubes, (B, C, E, and F) contained radioactive sodium acetate to the extent of approximately 0.01 millicurie per tube. It should be pointed out that the results in Table IV are expressed as counts per minute per aliquot of sample and bear no relationship to specific activity. The results in Table IV, obtained from samples prepared from 5 ml

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Experiment involving radioactive sodium acetate:

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TABLE III

Weight of Recovered Fatty Acids from

Experiments 1, 2 and 3

Experiment	Tube No.	Acetate Content in mg	Biotin in ug	Recovered Fatty Acids in mg
1	A*	-	-	45
	B*	5	-	219
2	A*	-	-	358
	B*	5	-	168
	C*	25	-	147
3-0 (A synthetic non-ionic oleate).	A*	-	-	52
	B*	5	-	168
	C*	25	-	84
	D#	-	-	32
	E#	25	-	203
	F#	25	0.03	187

* Cells grown on a synthetic medium containing biotin.
\# Cells grown on a synthetic medium containing Nopalcol 6-0 (a synthetic non-ionic oleate).

All tubes were incubated with 5 ml of cells, 5 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution and made to a volume of 17 ml.
Incubation was carried out for 2 hours at 37°C.

TABLE IV

Activity of Recovered Fatty Acids from Experiment 4

Tube No.	Acetate Content in mg		Biotin in ug	Counts per minute per aliquot above background	
	Non-Active	Active		0.5 ml	5 ml
A*	-	+	-	0	0
B*	4	1	-	6	17
C*	24	1	-	5	31
D#	-	+	-	0	0
E#	24	1	-	5	21
F#	24	1	1	10	19

* Cells grown on a synthetic medium containing biotin.
\# Cells grown on a synthetic medium containing Nopalcol 6-0 (a synthetic non-ionic oleate).

All tubes incubated with 5 ml of cells, 5 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution and made up to a total volume of 17 ml.

Incubation carried out for 2 hours at 37°C.

aliquots, were an attempt to raise the counting rate above that of the samples prepared with 0.5 ml aliquots. Although the counts here were somewhat higher than in the first count, there was considerable lumping of the oils on the watch glass and it was felt that there was too much self-absorption for these data to be of any value.

Other samples were prepared by placing 0.25 ml aliquots of the sample on small filter paper discs in one inch watch glasses and evaporating the solvent. This is standard procedure in counting certain types of samples but in this particular case the oils collected in a ring on the periphery of the disc and it was felt that the counts obtained were not representative of the sample.

In experiment 5 (Table V) the concentration of radioactive acetate was increased from 0.01 millicurie per tube to 0.1 millicurie per tube. It was thought that this higher level of activity would give higher counts and permit the counting to be carried out on smaller samples, and thus minimize the effects of absorption. When this failed to yield samples whose counts were significant, it was decided to set up an experiment and examine each liquid fraction in order to determine whether the acetate was being utilized, and, if so, to ascertain into which fraction or fractions the activity was going. The results of two such experiments are summarized in Table VI. Since the ether soluble fraction was so highly active, particularly in the 4 hour incubation, a count was made to determine the activity of the ether soluble fractions from Experiment 5, the findings of which are recorded in Table VII.

TABLE V

Activity of Recovered Fatty Acids from Experiment 5

Tube No.	Content of Active Acetate in mg	Biotin in ug	Counts per minute per aliquot above background
A*	-	-	0
B*	8	-	2.6
C#	-	-	0
D#	8	-	1.8
E#	8	1	1.2

* Cells grown on a synthetic medium containing biotin.
\# Cells grown on a synthetic medium containing Nopalcol 6-0.

All tubes contained 5 ml of cells, 5 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution and were made up to a total volume of 17 ml prior to incubation.

Incubation was carried out for 2 hours at 37°C.

TABLE VI

Activity of Various Fractions from Experiments 6 and 7

Experiment	Fraction	Content of Active Acetate in mg	Incubation Time in hours	Counts/minute/ml times total volume of fraction
6	1. Ether soluble*	8	4	32,587
	2. Alkaline supernatant**	8	4	6,500
	3. Fatty acid***	8	4	not significant
7	1. Ether soluble*	4.8	3/4	255
	2. Alkaline supernatant**	4.8	3/4	7.1×10^6
	3. Fatty acids***	4.8	3/4	not significant

* This fraction corresponds to the total ether solubles after saponification.
** This fraction corresponds to the alkaline supernatant left after copper-lime precipitation.
*** This fraction represents the petroleum ether solubles after the final acidification of the copper-lime precipitate.

In both runs the tubes were incubated with 5 ml of cells, 9 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution and made up to a total volume of 17 ml.

Cells in both runs were cultivated on a complete medium, containing liver extract, yeast extract, glucose and sodium acetate.

TABLE VII

Activity of Ether Soluble Fractions from Experiment 5

Tube No.	Content of Active Acetate in mg	Biotin in ug	cpm per ml times total vol of fraction
A*	-	-	0
B*	8	-	116
C#	-	-	0
D#	8	-	55
E#	8	1	225

* Cells grown on a synthetic medium containing biotin.
\# Cells grown on a synthetic medium containing Nopalcol 6-0 (a synthetic non-ionic oleate).

All tubes incubated with 5 ml of cells, 5 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution and made up to a total volume of 17 ml.

Incubation was carried out for two hours at 37°C.

A third experiment with radioactive acetate was undertaken in which the incubation period was 12 hours (Table VIII). This experiment confirmed the trend which was established in experiments 6 and 7, namely, that the longer the period of incubation the higher the count in the ether soluble fraction, the fatty acid fractions showing significant amounts of activity only in tubes B and C, which contained cells grown on a synthetic medium.

TABLE VIII

Activity of Various Fractions from

Experiment 8

Tube No.	Activity of Sample Fractions in cpm		
	Ether Soluble	Alkaline Supernatant	Fatty Acid
A*	101,150	1.41×10^7	not significant
B**	20,725	1.45×10^7	610
C***	17,825	1.42×10^7	730

* Cells grown on complete medium (see Table IV).
** Cells grown on synthetic medium containing Nopalcol 6-0.
*** Cells grown on synthetic medium containing Nopalcol 6-0. 1 ug of biotin added prior to incubation.

All tubes contained 5 ml of cells, 3 ml of pH 7.0 phosphate buffer, 1 ml of a sodium carbonate solution, 8 mg of radioactive acetate and were made up to a total volume of 17 ml prior to incubation.

Incubation was carried out for 12 hours at 37°C.

DISCUSSION OF RESULTS

In the preliminary experiments involving non-radioactive acetate (Table III), the weight of fatty acids obtained was quite variable. In one case, Experiment 2, the weight of fatty acids obtained was considerably higher in the control than in either of the tubes containing acetate. It would appear, however, that this value was erroneous, especially in view of the fair agreement obtained between the controls in Experiments 1 and 3. Otherwise, though, there was no correlation between either the level of acetate or the biotin content of the incubation mixture and the weight of fatty acids obtained. Since the incubation mixture was a complex system and in as much as acetate can enter into a number of cellular reactions other than synthesis of fatty acids, it is conceivable that the different conditions in the various tubes might lead to different degrees of utilization of the available acetate. Similarly, if biotin were involved in the synthesis of only one or several of the long chain fatty acids, its presence might not be expected to alter significantly the weight of the isolated fatty acid mixture.

On the strength of these data, work with non-radioactive acetate was abandoned in favor of the radioactive material. The results of the first experiment employing radioactive acetate are reported in Table IV. The first attempt to count the fatty acids obtained in

this experiment yielded counts which were insignificant in view of the extent to which background itself varied. Further samples, using 5 ml instead of 0.5 ml aliquots, yielded slightly higher counts. In this series of counts, tube C (Table IV) counted approximately twice as high as tube B. Although this seems in line with the increased level of acetate in tube C over tube B, it should be pointed out that in the latter tube 1/5 of the acetate present was radioactive (1 mg in 5 mg), whereas 1/25 of that in the former was active (1 mg in 25 mg). The activity of tubes E and F, which contained cells grown on Nopalcol 6-0, with and without added biotin, was approximately the same. Thus, if these results can be considered representative, it would appear that biotin had no effect on the activity of the isolated fatty acids. In these samples, however, there was considerable lumping of the oily mixture and because of the effects of self absorption the relationship between the various samples was of doubtful significance. Also the previously offered suggestion might be applied here: i.e., if biotin were involved in the synthesis of only one or several fatty acids of the mixture then its presence would not be expected to alter considerably the activity of the total mixture. Thus, it appears that if any interpretations are possible from these results, concerning the effect of biotin on the utilization of acetate for fatty acid synthesis, they will have to await isolation of the individual fatty acids.

Experiment 9 (Table V) in which the level of active acetate was increased approximately tenfold and in which no non-radioactive

carrier was included gave results as variable and of as doubtful significance as those in Experiment 4.

Since so little of the included activity seemed to be concentrated in the fatty acid fraction, two exploratory experiments were set up in which each liquid fraction was retained in a separate flask so that its activity might be determined. The results of these experiments, reported in Table VI, indicate that the majority of utilized active material was concentrated in the ether-soluble fraction. For the 45 minute incubation period the ether soluble fraction contained very little activity, considerable activity residing in the alkaline supernatant, indicating that very little of the acetate was utilized in such a short incubation period. For the 4 hour incubation period the activity of the ether-soluble fraction was considerably higher and the alkaline supernatant considerably lower, indicating greater utilization of the acetate; however, the activity of the fatty acid fraction was still insignificant.

Agreement with the exploratory experiments is found in the ether soluble fractions from Experiment 5 (Table VIII), when it is considered that the cells in this experiment were grown on a synthetic medium instead of a complete medium and hence would not be expected to synthesize as much active material in the same incubation period as cells grown on a complete medium. There seems to be some correlation in this fraction between activity and biotin content of the various tubes; however, it is not known if these results are reproducible.

The results of a 12 hour incubation with cells grown on complete and synthetic media are recorded in Table VIII. With cells grown on a complete medium the ether soluble and alkaline fractions were very active, whereas the fatty acid fraction showed no significant count. Tube B, which contained cells grown on Nopalcol 6-0 with no added biotin, showed activity in all fractions. The ether-soluble fraction, in agreement with the values in Table VII was considerably lower for cells grown on a synthetic medium than on a complete medium. Tube C which contained cells grown on Nopalcol 6-0 with biotin added to the incubation mixture showed less activity in the ether-soluble fraction than did tube B, this relationship being reversed for the fatty acid fractions.

The activity of the fatty acid fractions from tubes B and C have been redetermined and found to be significant, although there is some doubt as to the significance of the difference between the tubes. These values seem to explain the puzzling observation that in the exploratory experiments (6 and 7) with incubation times of 45 minutes and 4 hours and using cells grown on complete media, no activity could be found in the fatty acid fractions, while in Experiments 4 and 5 with an incubation time of 2 hours and using cells grown on synthetic media, a slight activity was noted in these fractions. These results would further seem to indicate that cells grown on a complete medium synthesized considerably larger amounts of non-saponifiable, ether-soluble substances and considerably less fatty acids when incubated in the presence of

acetate. This observation lends weight to the previously expressed opinion that under the different conditions of the incubation mixtures the cells can utilize the available acetate to varying extents for the synthesis of fatty acids.

It is interesting to note in Table VIII that, of the theoretical 2.22 times 10^8 dpm for 0.1 millicurie, 1.4 times 10^7 cpm are accounted for in the alkaline supernatant. While this value represents only 5 per cent of the theoretical amount of added activity a number of factors would indicate that actually this value should include much more on a dpm basis than is indicated by the cpm. In the first place the geometry of the counter would account for at least another 5 per cent. Add to this the effects on the initial count of self absorption and other uncontrollable factors which tend to reduce the correlation between dpm and cpm, as well as the fact that there are unreported 6 wash solutions which contain some activity, the difficulty of accounting for the theoretical amount of activity then becomes apparent. However, because of the insignificant amount of activity in the ether-soluble and fatty acid fractions as compared with 1.4 times 10^7, it can be seen that by far the majority of the activity resided in the alkaline supernatant.

SUMMARY

A significant synthesis of fatty acids from acetate has been demonstrated in several experiments and evidence has been presented which demonstrates that under different conditions the ability of cells of Lactobacillus casei to utilize acetate varies quite markedly. Thus, cells grown on a complete medium for 45 minutes, 2 hours and 12 hours failed to produce significant amounts of fatty acids from acetate, whereas detectable amounts of fatty acids were produced from acetate after 2 or 12 hour incubations in the presence of cells grown on a synthetic medium.

In a survey of the various fractions obtained in the course of isolating the long chain fatty acids it has been pointed out that most of the utilized radioactive acetate has been incorporated into the non-saponifiable, ether-soluble fraction, which fact is in agreement with many investigators. That very little of the included acetate is utilized is indicated by the very high level of activity of the alkaline supernatant; however, this fraction would include, in addition to any unutilized acetate, any saponifiable material including fatty acids of chain length shorter than 10 carbon atoms, which might have been synthesized.

From preliminary experiments using non-radioactive acetate as well as those involving the radioactive material it appears, as had been anticipated, that before any conclusions can be drawn concerning

the effects of biotin on acetate utilization for fatty acid synthesis, the crude fatty acid mixtures will have to be resolved. In fact, this resolution of the long chain fatty acid mixtures on a micro scale is a fundamental precept in the larger problem of which this work is a part.

On the basis of the data presented here it would be interesting in future research to determine whether a 24 hour incubation period would yield a fatty acid fraction of higher activity. Likewise, oxidation of the fatty acid mixtures and counting the evolved CO_2 by means of the vibrating reed electrometer should help to determine a relationship between the samples.

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VITA

James Thomas Jackson was born in Ponchatoula, Louisiana in 1926 and received his elementary and secondary education in the public schools there.

In 1943 he entered Louisiana State University but resigned in 1944 in order to enter the armed forces. After release from active duty, he reentered Louisiana State University in 1947. In July of the same year he was married to the former Shirley Ann Campbell.

In 1949 he received the degree of Bachelor of Science in Chemistry. The following semester he entered Louisiana State University as a graduate student and has since been engaged in a course of study leading to the degree of Master of Science in Biochemistry.