THE BIOSYNTHESIS OF FATTY ACIDS IN FLAX EMBRYOS, *LINUM USITATISSIMUM* L.

by

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A Dissertation Submitted to the Committee on

AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

UNIVERSITY OF ARIZONA

1961

THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Leslie F. Smith

entitled The Biosynthesis of Fatty Acids in Flax Embryos, Linum usitatissimum L.

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Edwin B. Kurtz Jr. — Dissertation Director — Sept. 5, 1961 — Date

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ACKNOWLEDGMENTS

Sincerest appreciation for the guidance and counsel of Dr. Edwin B. Kurtz, Jr. is gratefully acknowledged by the author. Dr. Kurtz's helpfulness and valuable technical advice contributed so much to this work.

The author is also thankful for the encouragement and ideas of Dr. Arthur S. Kemmerer, Dr. Mitchell G. Vavich, Dr. Walter S. Phillips, and Dr. Stanley M. Alcorn.

Special credit is given to the Department of Botany, University of Arizona, whose facilities were generously offered for this work.

My colleagues, Mr. Kaoru Matsuda, Mr. John McCarthy, and Dr. Nadim Abdul-Wahab deserve thanks for enjoyable hours of enlightening and stimulating discussions.

Without the financial assistance of the United States Atomic Energy Commission Contract No. AT (11-1)-262 to the Department of Botany and a Research Assistantship in the Department of Agricultural Biochemistry, this work would not have been possible.

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GENERAL INTRODUCTION

A substance is derived from the oil seeds of the plant kingdom that is highly important to man, but poorly understood by him as to its origin. Of this oil derived from plants, the unsaturated fatty acid components, oleic and linoleic acids, account for about 80 per cent and other unsaturated fatty acids account for 10 per cent. The saturated fatty acid components, mainly palmitic acid, make up less than 10 per cent of the total fatty acids produced in the seed oils of the world (18).

A general theory of plant fat biosynthesis must explain the appearance of the unsaturated fatty acid components as well as take into account the small but definite percentages of the saturated fatty acids. Since most animals cannot synthesize the common polyunsaturated fatty acids, the source of polyunsaturation must ultimately be from plants. Research on the mechanisms of unsaturation logically begins with plants.

There are two theories to account for the formation of unsaturated fatty acids in developing seeds: 1, saturated fatty acids are first formed and then dehydrogenated; 2, the individual fatty acids are formed by different mechanisms. Investigators do not agree upon either mechanism.

In the past, research on the biosynthesis of fatty acids in plants has centered on analysis of the component fatty acids of the

oil during seed development. Apart from growing various oil seed plants under different climatic conditions and observing the differences in the unsaturated character of the oil, very little experimental work has been done.

Kurtz and co-workers (16, 20, 21) helped establish an experimental basis for fatty acid research in plants. Working with the flax fruit and later with the flax embryo, they found that the fatty acid composition of the developing flax oil could be altered experimentally. These workers observed that flax embryos cultured *in vitro* synthesized more linoleic and linolenic acids when supplied with biotin and light.

In extension of the previous studies of Kurtz *et al.*, this work will deal with the action of biotin and light in the synthesis of fatty acids, particularly polyunsaturated acids, in developing embryos of flax and will be presented in three parts.

Part I The physiological relationships of biotin and lipides.

Part II The influence of light on the synthesis of lipides and fatty acid components.

Part III Studies on the pathways of biosynthesis of the fatty acids.

Part I

The Physiological Relationships of Biotin and Lipides

INTRODUCTION

The role of biotin in carbon dioxide metabolism points to a close relationship between this vitamin and carboxylation-decarboxylation reactions. In 1927 Boas (6), observed that rats which suffered severe biotin deficiency possessed almost no stores of body fat. Gavin and McHenry (15) obtained fatty infiltration of the liver and an increase in the body fat of rats by administration of biotin. Williams et al. (9, 56) pointed out that biotin deficient cells of Lactobacillus arabinosus could grow if oleic acid was supplied instead of biotin. They postulated that in those organisms for which biotin and oleic acid are mutually replaceable, biotin functions in the synthesis of that acid.

The direct involvement of biotin in fatty acid synthesis was first shown by Kurtz and Miramon (21, 30). This vitamin stimulated the incorporation of radioacetate into lipides of developing flax fruits (20) and flax embryos cultured in vitro. In the flax embryo, biotin stimulated the in vitro synthesis of long-chain fatty acids, especially linoleic and linolenic acids, from acetate-1-C^{14}.

Wakil et al. (51, 52, 53, 54, 55) obtained a supernatant from a pigeon liver homogenate. This supernatant was fractionated into R_1 and R_2 by precipitation with ammonium sulfate. The mixture of these two enzyme fractions catalyzed the anaerobic conversion of acetyl-CoA to long-chain saturated fatty acids, mainly palmitic,

when supplemented with ATP, CO_2, Mn^{++}, and TPNH.[1] Fraction R_1 contained high concentration of bound biotin (1 mole/3-5 x 10^5 g of protein) (51, 55). This enzyme was inhibited by avidin, a known biotin inhibitor, and this inhibition was overcome by addition of biotin in catalytic amounts.

It was postulated that enzyme-bound biotin combined with CO_2 in the presence of Mn^{++} and ATP, and that the activated CO_2 was transferred to the α-carbon of acetyl-CoA with the formation malonyl-CoA (53). Malonyl-CoA was rapidly decarboxylated in the presence of the R_2-fraction, but acetyl-CoA as such did not enter into fatty acid synthesis except in the presence of malonyl-CoA. Butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA were also capable of entering into the reaction with malonyl-CoA.

Fischer reported that mitochondria from biotin deficient rats failed to convert isovalerate, β-methyl crotonate, or β-methyl-vinyl acetate to acetoacetate (13). Woessner et al. (57) found that β-hydroxyisovaleryl-CoA carboxylase was completely lacking in biotin deficiency. Biotin enzymes catalyze the carboxylation of the α-carbons of acetyl-, propionyl-, and butyryl-CoA to form the corresponding malonyl, α-methyl-malonyl, and α-ethyl malonyl-CoA derivatives (43). In another biotin carboxylation reaction, Lane et al. (22)

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[1]Abbreviations used in this dissertation are as follows:

ATP	Adenosine triphosphate
CoA	Coenzyme A
DPN & DPNH	Diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide
TPN & TPNH	Triphosphopyridine nucleotide and reduced triphosphopyridine nucleotide

pointed out that in rat liver, the α-β unsaturated derivative β-methyl crotonyl-CoA, was carboxylated on the γ-carbon to form β-methyl glutaconyl-CoA. The vinylog conjugation produced by the α-β unsaturation center probably shifted the nucleophilic center from the α- to the γ-carbon.

Although biotin had been shown to be involved in different carboxylation reactions in the metabolism of both short-chain saturated and short-chain unsaturated fatty acid CoA derivatives, very little was known of its action in the synthesis of long-chain fatty acids, especially the unsaturated fatty acids.

Plant tissues have been observed to contain biotin. Developing flax embryos were assayed for this vitamin to determine the relation of biotin in the parts of flax fruits and fatty acid synthesis in the embryos.

METHODS AND MATERIALS

A. Greenhouse culture of flax embryos. Flax (Linum usitatissimum L., var. Punjab) was grown in the greenhouse. The flowers were marked by tagging each peduncle with a piece of colored cellophane tape. In this way, fruits of the same age in days could be collected (16). Fruits were harvested from 10 to 30 days after flowering, depending upon the experiment to be performed. Fruits were harvested by cutting, immersed in distilled water and used immediately. A 2-mm slice was made through the base of the fruit exposing the tips of the cotyledons of the embryos (30). The fruits were squezed and the embryos were extruded into a filter funnel and paper and washed with distilled water. The distilled water was drained off and the washed embryos were subjected to biotin assay, fat analysis, or in vitro culture.

B. Biotin assay. Lactobacillus arabinosus was used for the microbiological assay of this vitamin after the method of Wright and Skeggs (58). The procedure followed was a modification of that outlined in the Methods of Vitamin Assay (29). In these experiments, the necessity of preparing a culture agar and an assay medium was eliminated by use of a Difco prepared agar medium. The assay procedure was further modified in the use of only freshly incubated stock cultures for preparation of the inoculum, and the use of freshly

incubated inoculum for the inoculation of the standard and assay tubes. In addition, screw-cap culture tubes were used throughout the assay. The standard error of the method was found to be 4 per cent. The detailed procedure is described in the Appendix of Methods, Part I.

C. Analysis of the biotin assay method. Ten replicates of one concentration of biotin were run as standards. Similarly, 10 replicates were run of a flax embryo extract in this experiment. The results are shown in Table 1. The average error of the method was ± 4.0 per cent. Biotin assay values that differ by more than 8 per cent each are significantly different at the 5 per cent level.

Duplicate samples of 17 and 18-day flax embryos were subjected to biotin analysis (Table 2).

D. Fat content of flax embryos. Embryos were collected and dried at 60°C for 4 hours. The dried material was macerated and the fat was extracted into petroleum ether (d. 0.67-0.69). The fat was weighed and reported as mg/100 embryos.

E. In vitro culture system. The in vitro culture system was similar to that used by Kurtz and Miramon (21). They showed that flax embryos were more effective than the whole flax fruit for incorporation of radioacetate into lipides. In this study, about 50 freshly-washed flax embryos were placed in open-end roller culture tubes, 4 cm in length by 2 cm in diameter. Each tube contained 0.5 ml of culture

Table 1. Statistical analysis of the biotin assay method. I. Titration values.

Assay Material	ml 0.1 N NaOH
Biotin standard (0.1 mμg/tube)	7.20 ± 0.26*
Embryo extract	5.02 ± 0.22

*Standard deviation

Table 2. Statistical analysis of the biotin assay method. II. Embryo biotin content.

Age	Embryos		Biotin	Biotin/
	Weight	Number Assayed		100 Embryos
days	mg		mμg	mμg
17a*	95.2	100	42	42
17a	100.0	100	44	44
17b*	63.9	100	42	42
17b	63.7	90	40	44
18	58.3	100	28	28
18	63.3	100	28	28

*17a and 17b represent different experiments.

solution which had 100 mg Na-acetate-1-C^{14} (1 mc/mM) and 10 µg biotin/l of 0.1 M phosphate buffer, pH 6.0. The culture tubes were rotated at 3 rpm on a horizontal rotary culture apparatus. The cultures were maintained for 24 hours in a controlled growth chamber at 15°C. The light source was a bank of cool daylight fluorescent tubes.

F. Extraction of lipides. After each culture period, the embryos were dropped into a 50-ml erlenmeyer flask containing about 30 ml of boiling petroleum ether (d. 0.67-0.69). The embryos were counted, macerated, and the lipides were extracted into the petroleum ether by refluxing under an air condenser for 4 hours. The petroleum ether extracts were washed 5 times with 10-ml portions of 1 per cent $NaHCO_3$ to remove unincorporated radioacetate from the lipide extracts. The washed extracts were placed in a tared 100-ml volumetric flask and made up to volume. Aliquots of 0.5 ml were plated in 3 replications on 2.5-cm copper planchets and dried on the hot plate.

Total activity of each lipide extract on the planchets was determined using a windowless flow counter (Tracerlab Model SC-16). The specific activity of the samples was calculated from the activity (minus background) and the total weight of the lipide remaining in the volumetric flask after evaporation of the petroleum ether on a hot plate.

G. Analysis of the specific activity method. Cultures of 16-day flax embryos were run in duplicates for 1, 2, 4, and 8 hours. Each

culture contained approximately 50 embryos, 10 µg biotin/l and Na-acetate-1-C^{14} (1 mc/mM; 2000 mg/l) in 0.5 ml 0.1 M phosphate buffer, pH 6.0. The embryos were placed in a controlled temperature and light chamber at 15°C and 1200 ft-c light intensity.

After culture, the lipides were extracted and the washed extracts were plated onto copper planchets in 3 replications. The results are shown in Table 3. The average error of the analysis was ± 12.4 per cent. Specific activity values that differ by more than 20 per cent are significantly different at the 10 per cent level.

Table 3. Statistical analysis of the specific activity method.

Time hours	Specific Activity dpm/mg fat
1	2150 ± 410*
2	1732 ± 232
4	1694 ± 163
8	1971 ± 143

*Standard deviation

RESULTS AND DISCUSSION

A. Biotin analysis of flax. Biotin assay of the flax plant was undertaken (Table 4). The highest concentration of biotin occured in the flax embryo. During the early stages of development of the embryo, the biotin content was low and increased as maturation progressed. After a maximum point was reached, the biotin content decreased slightly in amount. Analysis of the parts of the fruit (Tables 5 and 6) shows that the biotin content of the seed coats and fruit walls continued to increase during development.

Tables 5 and 6 and Figure 1 (May, 1959) show analyses of the same embryos. The embryos, which averaged about 8 per fruit, were removed from the fruit and were collectively analyzed, as shown in Table 5. The seed coats and nucelli surrounding each embryo, also 8 per fruit, were analyzed as a group. The average biotin content for individual embryos and seed coats are reported in Table 6.

The environment at which the embryos mature influenced the biotin content. Although no precise measurements were recorded, Figure 1 illustrates the variability of the biotin content of embryos grown under different environments. These studies emphasize the need of a controlled environment greenhouse for obtaining uniform plant material.

Embryos grown in April, 1959 and 1960, show good uniformity as to dry weight and biotin content at each age collected. In these

Table 4. Biotin content of flax plant parts. The fruits were grown during March, 1959.

Plant Part	Biotin Content
	mμg/g dry weight
Complete flowers	370
Leaves	490
16-Day flax fruits	
Sepals	550
Fruit walls	530
Seed coats	530
Embryos	1,200

Table 5. Biotin content of parts of maturing flax fruits. The fruits were grown during April, 1959.

Age of Fruit	Biotin/Fruit Part of One Fruit		
	All Embryos	All Seed Coats	Fruit Wall
days		mμg	
11	1.2	3.2	3.0
14	5.0	4.8	4.6
15	4.0	4.6	3.6
16	5.2	5.2	4.8
17	3.8	4.8	4.6
19	3.1	4.2	5.8
21	2.4	9.0	10.0

Table 6. Biotin content per part of a maturing flax fruit. The fruits were grown during April, 1959.

Age of Fruit	Biotin/Fruit Part of One Fruit		
	Embryo	Seed Coat	Fruit Wall
days		mμg	
11	.15	.39	3.0
14	.59	.57	4.6
15	.52	.60	3.6
16	.61	.61	4.8
17	.49	.62	4.6
19	.42	.56	5.8
21	.30	1.14	10.0

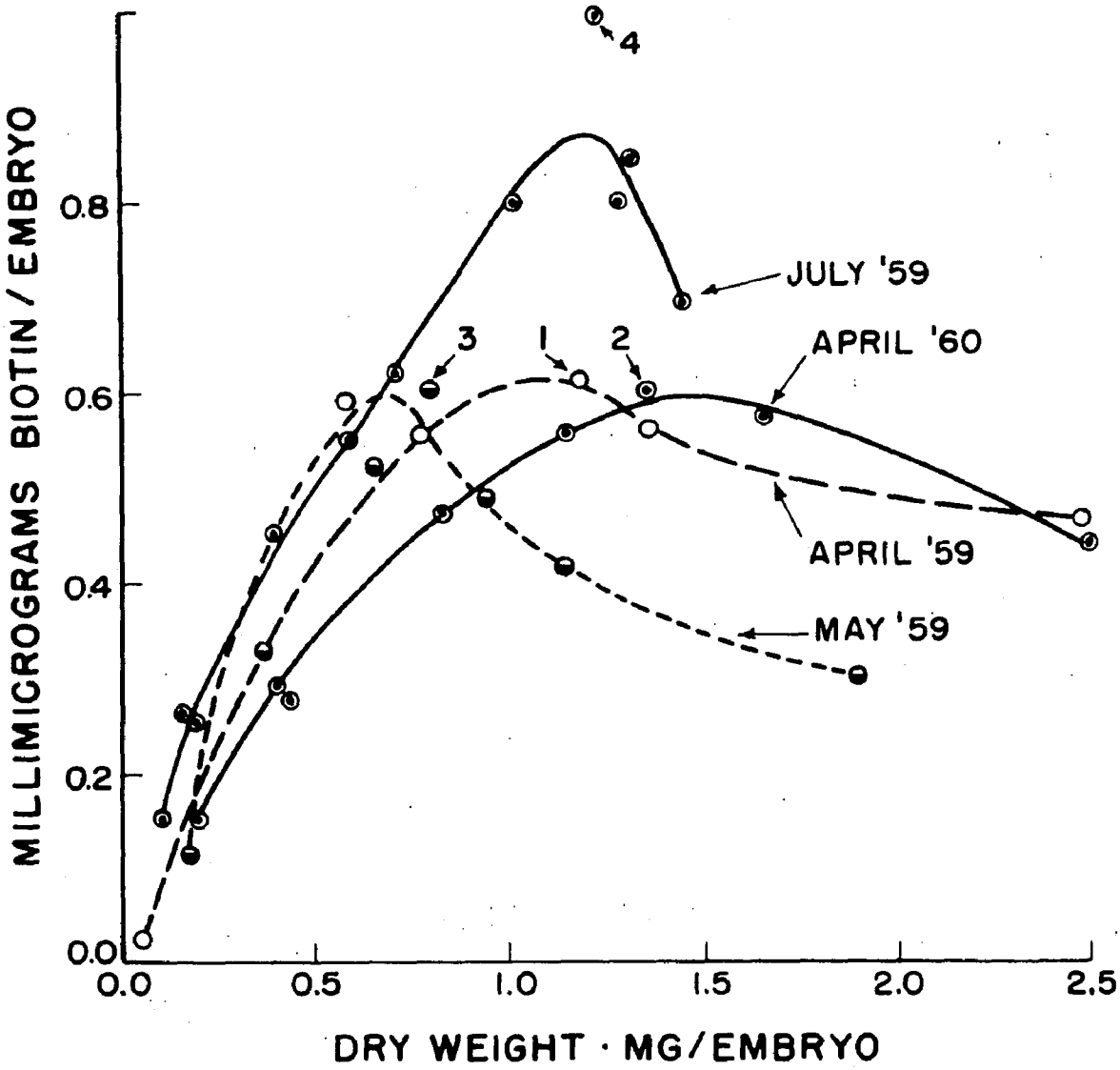

Figure 1.
Biotin content of maturing flax embryos grown during different months. The number of each curve indicates maximum biotin content. The ages at which these maxima were attained are: 1, 19 days; 2, 20 days; 3, 16 days; 4, 17 days.

analyses, the maximum biotin content was reached at about 20 days of age. In the embryos grown during the warmer months, the maximum biotin content occurred 16 days after flowering in the May analysis and after 17 days in the July analysis. In July the maximum biotin content was approximately 50 per cent higher than in other months. The maximum biotin content which was obtained during the months of April and May, approximately 0.6 mμg biotin/embryo, was obtained in only 15 days in embryos grown during July.

In an experiment to determine the site of biotin synthesis, 13-day fruits were removed from the plant and either whole fruits or fruit parts were cultured in acetate (100 mg/l in 0.1 M phosphate buffer, pH 6.0) for 2 days. Some fruits were left on the plant for 2 days as a control. After the 2-day period, the control and cultured fruits were divided into embryos, seed coats (and nucelli) and fruit walls. Each part was subjected to a biotin assay. Results were unclear because the biotin content of the seed coats and fruit walls decreased after 2 days of culture both on and off the plant. The biotin content of the embryos doubled in fruits left on the plant, but the biotin content of the embryos of cultured fruits did not increase. Similarly, embryos removed from the fruits and cultured in acetate showed no synthesis of biotin. It appears that biotin is not synthesized in the embryo, but since the surrounding fruit tissues seem to be a rich source of biotin (shown also in Tables 5 and 6), it is thought that biotin may be synthesized in the surrounding fruit parts and then may diffuse into the embryo.

B. The effect of biotin on lipide synthesis. It was necessary to find a good criterion of physiological age of the embryos so that the fat synthesis could be related to the age during maturation. Chronological age, size of the embryo, and dry weight were all used at various times. It was found that the dry weight of the embryo was the best indicator of the physiological age of the embryos.

The effect of biotin on the synthesis of lipides as reported by Miramon (30), was confirmed (Table 7). In these experiments, biotin was most effective in 19-day embryos, as measured by the specific activity of the lipides.

Figure 2 shows the biotin content, fat content and specific activity for developing embryos from 10 to 20 days of age. The curve of the biotin content shown in Figure 2 is the same as the curve shown in Figure 1 for July, 1959. Figure 2 shows that the rise in biotin content preceeded fat synthesis in the flax embryo and that the rate of fat synthesis is related to the biotin concentration. Addition of biotin to the embryos cultured in vitro in acetate greatly increases the rate of fat synthesis, especially if the embryos are of a size to contain moderate or low amounts of endogenous biotin. Introduction of biotin into the culture medium presumably increases the biotin concentration of these embryos to a maximal level that occurs normally in larger and older embryos (Table 7). When the biotin concentration is brought to such a level, then fat synthesis is most rapid, as shown by the specific activity peak. Introduction of biotin into cultures of older and larger embryos likewise

Table 7. Effect of biotin on the incorporation of radioacetate into lipides of maturing flax embryos cultured *in vitro*. Embryos were cultured for 24 hours in Na-acetate-1-C^{14} (1000 mg/l, 1 mc/mM) in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensity, 1,200 ft-c; temperature, 15°C. Values shown in Experiments I and II are obtained from embryos analyzed in Figure 1 for April, 1959 and May, 1959, respectively.

Embryo		Specific Activity	
Age	Weight	Without Biotin	With Biotin
days	mg/embryo	counts/minute/mg fat	
Experiment I			
10	0.06	1,081	199
15	0.37	919	100
17	0.77	1,688	1,682
19	1.20	72,463	121,638
20	1.34	51,411	57,573
Experiment II			
11	0.19	145	294
14	0.60	1,903	1,026
15	0.65	1,185	1,169
16	0.82	1,449	2,283
17	0.95	1,155	2,319
19	1.16	3,653	10,518
21	1.90	14,461	15,388

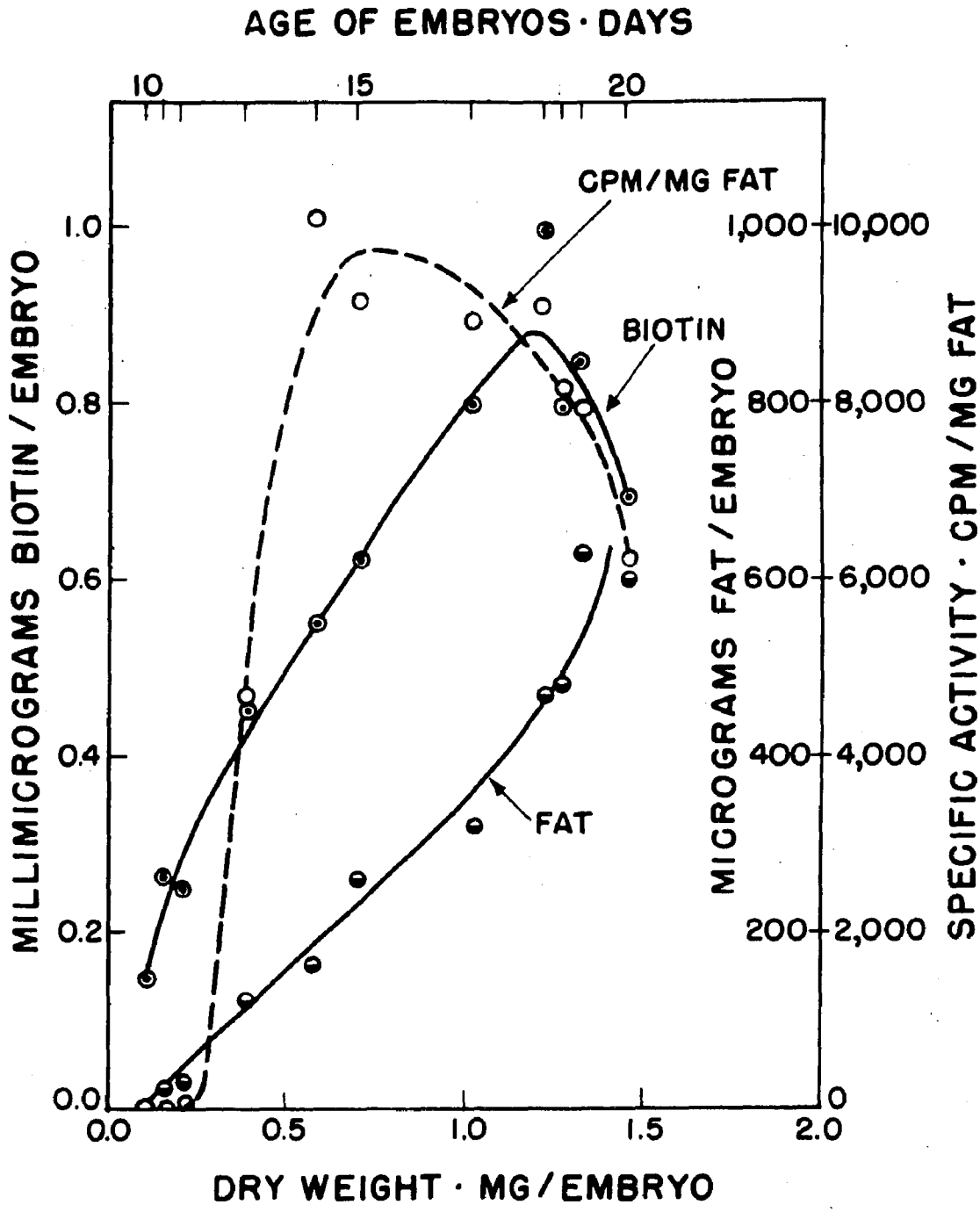

Figure 2.
Relationships between biotin content, fat content and specific activity of maturing flax embryos. Values are shown daily from 10 to 20 days. Embryos were cultured for 24 hours in Na-acetate-1-C^{14} (100 mg/l, 1 mc/mM) in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensity, 200 ft-c; temperature, 15°C. Embryos were from fruits grown during July, 1959. Biotin and fat curves indicate endogenous concentrations in the embryos.

stimulates fat synthesis, but the specific activity decreases slightly due to a dilution of the radioactive fats by endogenous non-radioactive fats in the embryos at the start of the culture period.

Addition of biotin to very small embryos does not increase fat synthesis presumably because the enzyme system necessary for fat synthesis is in low concentration or is incompletely present.

C. The effect of biotin and biotin analogues on lipide synthesis. The effect of biotin and biotin analogues on the synthesis of lipides in vitro in 15-day embryos is shown in Figure 3. D-biotin, the naturally occurring isomer, was the most active form; the specific activity of the lipides increased as the concentration of D-biotin increased. D-homobiotin was active but is somewhat less effective than D-biotin at lower concentrations. The other analogues seemed to act as inhibitors at 1000 µg/l. L-biotin was inactive; it was neither stimulatory nor inhibitory at any concentration. This experiment, however, has not been replicated and the results must be accepted as preliminary.

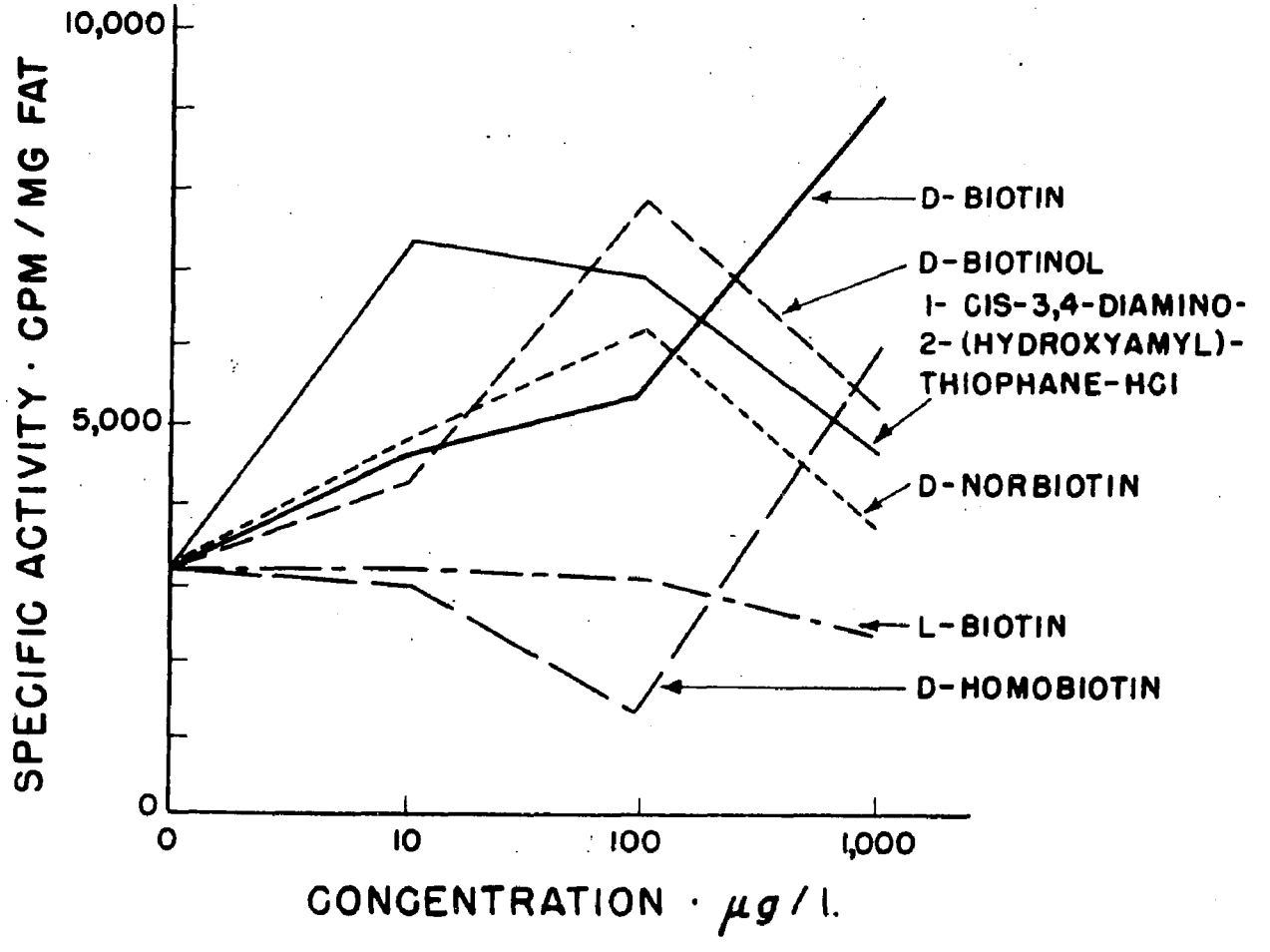

Figure 3.
The effect of biotin and biotin analogues on the incorporation of radioacetate into fats in fifteen-day flax embryos. Embryos were cultured for 24 hours in Na-acetate-1-C^{14} (100 mg/l, 1 mc/mM) in 0.1 M phosphate buffer, pH 6.0; light intensity, 200 ft-c; temperature, 18°C. The embryos were from fruits grown during July, 1960.

SUMMARY

1. Biotin analysis of developing flax embryos was performed using Lactobacillus arabinosus as the test organism.

2. Biotin was detected early in the development of the flax embryo. The biotin content rises sharply to a maximum and then decreases slightly in a period of about 10 days. The biotin content increases most rapidly and to the highest concentration in embryos grown during warm months.

3. The biotin content of 13-day embryos did not increase in excised fruits cultured for 2 days. The biotin content of the fruit walls and seed coats decreased after 2 days in fruits on the plant or in acetate culture. It is thought that the synthesis of biotin in the flax fruit is extraembryonic.

4. Biotin stimulates the in vitro synthesis of lipides from radioacetate in the developing embryos of flax.

5. Concomitant analyses of biotin content, fat content, and fat-synthesizing capacity were performed on developing embryos 10 to 20-days old. During embryonic development there is a certain physiological age, usually 14 to 17 days, when the endogenous biotin content is low and the synthetic capacity of the embryo is enhanced by addition of biotin in vitro.

6. D-biotin and D-homobiotin are active in stimulating the synthesis of lipides in vitro. L-biotin was inactive in 15-day embryos.

Part II

The Influence of Light on the Synthesis of Lipides and Fatty Acid Components

INTRODUCTION

Many photochemical reactions occur in plants which are necessary for their growth and metabolism. Light has been reported to be necessary for the synthesis of anthocyanin in the corn endosperm (45). Similarly, the synthesis of the red pigment of apple skins required light energy (37). The spectral region of 6000 to 7000 Å appeared to be optimum for this synthesis. Besides photosynthesis, other photochemically controlled reactions observed in plants were: photoperiodic control of flowering, dormancy of seeds, and cell elongation (49).

Apparently the role of red light in plant metabolism is of fundamental importance. In all photoreactions in which red light had a specific physiological effect, this effect could be reversed by far-red light (7350 Å). Abdul-Wahab (1) showed that the stimulatory effect of low intensity red light on fat synthesis could be reversed by far-red light. He stated that the metabolism of acetate in flax embryos was controlled by a light reaction, and indicated red and far-red light could shift the utilization of acetate to fat synthesis or respiration, respectively.

It has been observed that both biotin and light are necessary for the synthesis of the unsaturated fatty acids, linoleic and linolenic, in developing embryos of flax (21, 30). Without biotin and light supplied to the culture, the fatty acids synthesized are principally oleic and stearic.

There is the possibility that red and far-red light may control the synthesis of the component fatty acids of flax. The action of light, particularly red and far-red light, and the interactions of light quality and biotin on fatty acid synthesis are discussed in this part of the dissertation.

METHODS AND MATERIALS

A. In vitro culture systems.

1. Light intensity studies. The in vitro culture system has been described in Part I under section E of Methods and Materials. The roller culture tubes containing the flax embryos and culture solutions were placed on the rotary culture apparatus which was adjusted to rotate the culture tubes at different distances from the light source. In the lowest intensity light treatment (50 ft-c), the culture tubes were also shaded with three layers of white cheesecloth to give the desired light intensity. A Weston No. 603 photometer was used for intensity measurements.

2. Light quality studies. The in vitro culture system was similar to that described in Part I under section E of Methods and Materials. In this study, however, the washed flax embryos were placed in stainless steel, deep-well planchets, 2.5 cm in diameter. The planchets were set in 9-cm Petri dishes and the Petri dishes were placed in coffee cans. A 9-cm diameter hole was cut in the top of all coffee cans used except those which were used for cultures in the dark. The lids were replaced on the cans and covered with colored cellophane, to form a light filter. White light was obtained without cellophane filter from cool daylight fluorescent tubes. Red light was obtained from white fluorescent light filtered through two layers of DuPont No. 300 PC Red cellophane. Far-red light was

obtained by filtering the light from á 100-watt Mazda incandescent bulb through two layers each of red and blue (DuPont No. 300 PC dark blue) cellophane (2). Other cultures were kept under dark conditions in coffee cans fitted with tight lids. The temperature of all treatments was the same. Studies involving alternate treatments of red and far-red lights necessitated a change in light filters without disrupting the cultures in the coffee cans.

In another study, a planchet with embryos was placed in 6 individual 9.0-cm blackened bottom deep Petri dishes. The lid of each Petri dish was a different colored light filter mady by covering each lid with two layers of red, yellow, green or blue DuPont cellophane. The light source was cool daylight fluorescent tubes. Light and dark cultures had transparent and blackened Petri dish lids, respectively.

B. Chromatography of fatty acids.

1. Saponification procedure. Lipides were extracted according to the procedure described in Part I under section F of Methods and Materials. The lipide extracts were dissolved in 10 ml of 0.5 N KOH in 90 per cent methanol per 0.1 gram fat. The methanol and petroleum ether were removed by evaporation on a steam bath for 2 hours or until the odor of alcohol and petroleum ether was no longer detectable. When all the alcohol and petroleum ether had been removed, 10 ml of distilled water was added and the mixture allowed to stand at room temperature for 12 hours to insure complete saponification. The unsaponifiable material was extracted from the saponification

mixture with one 20-ml and one 10-ml portion of diethyl ether in a separatory funnel fitted with a Teflon stopcock. The aqueous layer was acidified with 6N HCl and the free fatty acids were extracted with one 20-ml and one 10-ml portion of diethyl ether. The combined ether extracts were dried over 10 g anhydrous $MgSO_4$ for two hours and filtered. The ether solution containing the free fatty acids was evaporated to a small volume and 0.1 ml of petroleum ether (d. 0.67-0.69) was added. This concentrated solution was used for chromatographic analysis.

2. Chromatograms. The chromatography strips were prepared as reported by Miramon, Kurtz and Smith (31). The procedure is described in the Appendix, Part II. The paper strips were impregnated with a stationary phase of paraffin oil, 90 g/l. Ten µl of the concentrated extract were spotted onto the prepared paper and the chromatograms were run for 30 hours in a solvent of 90 per cent acetic acid saturated with paraffin oil. The paper strips were removed from the chamber and air-dried for 3 hours.

3. Scanning for radioactivity. The radioactive chromatograms were connected end to end, placed in the paper strip scanner (Nuclear-Chicago Model 1620A) and scanned for activity. The radioactive fatty acids appeared as peaks on the chart, the areas of which were determined with a planimeter. The areas of radioactive fatty acid peaks were converted into total disintegrations per minute by comparison with the areas obtained by scanning serial dilutions of radioacetate (10 mc/mM). The total disintegrations per minute devided by the number of embryos in the culture gave the values

reported in the tables: disintegrations per minute per embryo.

The peaks on the scanning chart were correlated with Rf values of known fatty acids. Additional tests for the unsaturated fatty acids were performed by spraying the previously scanned paper strip with a solution of permanganate-periodate after the method of von Rudloff (50). The strips were washed in water. This procedure oxidized the unsaturated fatty acids and all oxidation products except nonanoic acid were removed by the water washings. The re-scanned strips showed no activity in the polyunsaturated fatty acid areas (see Appendix of Methods, Part II).

Three methods were used to identify the radioactive peaks on the strip-scanned radiochromatograms:

1. Correlation of the radioactive peaks with the Rf values for known reference fatty acids.

2. The brown spots appearing on the radiochromatogram as a result of unsaturated fatty acid oxidation during periodate-permanganate treatment were correlated with the Rf values of the radioactive peaks and known or reference fatty acids.

3. Rescanning of the paper strip to verify the oxidation of the unsaturated fatty acids and lack of oxidation of the saturated fatty acids.

Radioactivity remaining in the region containing oleic acid peak after oxidation indicates that the whole carbon chain was made up of radioacetate.

C. Analysis of the method of in vitro culture, fatty acid synthesis, and chromatography. In this experiment, 17-day flax embryos were cultured in Petri dishes in quadruplicate with and without the

addition of 10 µg biotin, as described in Part III under section A of Methods and Materials.

After extraction and saponification of the fatty acids, duplicate chromatograms were analyzed. Per cent composition of each fatty acid was determined by measuring the area beneath each curve with a planimeter and calculating: $\frac{\text{area beneath curve}}{\text{total area beneath all curves}} \times 100.$

The final results of experiments in Part III are reported as disintegrations per minute. In Table 8, however, results are reported as per cent fatty acids and per cent standard deviation. It is unnecessary to convert per cent fatty acid to disintegrations per minute for statistical analysis. The average error of the method was $\pm$ 10.9 per cent. Treatments that differ by more than 17 per cent are significantly different at the 10 per cent level.

Table 8. Statistical analysis of the method of *in vitro* culture, fatty acid synthesis and chromatography.

Treatment	Stearic	Oleic	Linoleic	Linolenic
		per cent		
Biotin + Light	5.9 ± 0.9*	27.1 ± 1.9	43.9 ± 2.9	23.1 ± 4.3
Light	6.5 ± 1.4	23.6 ± 2.3	45.0 ± 4.5	12.9 ± 3.5

*Standard deviation

RESULTS AND DISCUSSION

Table 9 shows the effect of light intensity of the synthesis of lipides in 16-day embryos. A light intensity of 100 ft-c. was best for incorporation of radioacetate into lipides, as measured by the specific activity.

The effect of light on the synthesis of lipides can be seen in the comparison of the white light and dark treatments (Table 10). Embryos receiving light during culture synthesized fat that was nearly 5 times more radioactive than embryos cultured in the dark. An action spectrum of light quality versus specific activity was run for 15-day embryos (Table 10). Approximately the same light intensity was used in all treatments. It was found that all wavelengths were as effective as cool white fluorescent light in stimulating incorporation of radioacetate into lipides.

It is suggested that one action of light is to provide energy for fat synthesis through a photosynthetic phosphorylation process. In a similar system using flax embryos, Abdul-Wahab (1) claimed to have synthesized fats from radioacetate that were as radioactive in dark cultures as in the light, by the addition of certain co-factors to the dark culture. These co-factors are TPNH and ATP, two products of photosynthetic reactions. Unfortunately, the component fatty acids were not analyzed in that experiment.

Table 9. Effect of light intensity on the synthesis of lipides in flax embryos. Sixteen-day flax embryos were cultured for 24 hours in Na-acetate-1-C^{14} (100 mg/l, 1 mc/mM) in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; temperature, 15°C. Embryos were from fruits grown during July, 1959.

Light Intensity	Specific Activity
ft-c.	counts/minute/mg fat
50	1,786
100	5,116
350	2,243
500	2,871
800	3,717

Table 10. Relative specific activity of lipides *versus* culture light quality for flax embryos. Fifteen-day flax embryos were cultured for 24 hours in Na-acetate-1-C^{14} (500 mg/l, 3.6 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C. Embryos were from fruits grown during July, 1960.

Light Quality	Light Intensity	Relative Specific Activity
	ft-c.	counts/minute/embryo
Dark	0	4,213
Blue	50	14,969
Green	30	19,685
Yellow	70	21,635
Red	50	22,704
White	200	20,550

Both specific activity (Table 10) and unsaturated fatty acid synthesis (Table 11) were enhanced by light treatment. Both radioactivity and degree of unsaturation were increased by light in the red region of the visible spectrum. It has been observed that, although light enhances the synthesis of stearic and oleic acids, the wavelength of light used in the culture has little effect upon this synthesis. The synthesis of the polyunsaturated fatty acids, on the other hand, is dependent upon the wavelengths of the culture light. White light stimulates synthesis of both stearic and oleic and the polyunsaturated fatty acids.

In another experiment (Table 12), far-red light (approximately 7350 Å) enhanced the synthesis of stearic and oleic acids, whereas the synthesis of linoleic and linolenic acids was again dependent on red light (approximately 6550 Å). Data on the transmission spectrum of the far-red filter (red and blue cellophane) shows that some red light is transmitted although the majority of the light is in the far-red region. It is thought that synthesis of stearic and oleic acids may be enhanced by the red light possibly by photosynthetic reduction of the necessary co-factors for fatty acid synthesis. The far-red light is thought to be ineffective in synthesis of stearic and oleic acids.

These data suggest that there are two physiological actions of light. One action is general and enhances the synthesis of all the fatty acid components and is independent of the wavelength with the possible exception of green light. The other physiological action of light is specific and enhances the synthesis of polyunsaturated fatty acids with increasing wavelengths in the visible

Table 11. Effect of light quality on the synthesis of the component fatty acids in flax embryos *in vitro*. These embryos are the same as those in Table 10. The fatty acids were obtained from the radioactive lipides reported in Table 10. The culture conditions are the same as in Table 10.

Light Treatment	Stearic	Oleic	Linoleic and Linolenic	Total
		disintegrations/minute/embryo		
Dark	35	46	6	87
Blue	118	432	148	698
Green	103	264	191	558
Yellow	120	433	348	901
Red	112	305	390	801
White	63	625	404	1,092

Table 12. The effect of light quality on the relative specific activity of the fatty acids of flax embryos cultured *in vitro*. Fifteen-day flax embryos were cultured for 16 hours in Na-acetate-1-C^{14} (500 mg/l, 3.6 mc/mM) in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensities, red 50 ft-c, far-red 20 ft-c; temperature, 25°C. Embryos were from fruits grown during November, 1960.

Light Treatment	Stearic	Oleic	Linoleic and Linolenic
	disintegrations/minute/100 embryos		
Dark	56	91	0
Far-red	220	220	0
Red	82	506	118

spectrum. Far-red light, however, completely inhibited synthesis of polyunsaturated fatty acids. This dual action of light indicates that there may be two pathways for the synthesis of fatty acids. One path may be for the synthesis of stearic and oleic acids, the energy for which may or may not be supplied by light. The other path may be necessary for the specific synthesis of polyunsaturated fatty acids. Since no polyunsaturated fatty acids appear during culture in far-red light (Table 12), it is thought that dehydrogenation of stearic and oleic acids to form linoleic and linolenic acids is not an important pathway in the biosynthesis of these polyunsaturated fatty acids.

The effect of light quality on the synthesis of lipides is also a function of embryo age (Table 13). Specific wavelengths had very little effect on physiologically young embryos, but had a greater influence on the lipide synthesis of physiologically old embryos. Young embryos synthesize predominately stearic and oleic acids (see Part III), and therefore the light requirement may be non-specific, as the data suggest. In 17-day embryos, red and far-red light did have an effect on lipide synthesis, but unfortunately the fatty acids were not analyzed. Assuming that all fatty acid components were made in the old embryos (see Part III), the data suggest that the rate of synthesis of polyunsaturated fatty acids must be slower than synthesis of stearic and oleic (Table 13). This rate of synthesis of the different fatty acids is arrived at from other data in Part III.

Table 13. Effect of red and far-red light on the synthesis of lipides in flax embryos. Embryos were pre-cultured for 6 hours in 0.1 M phosphate buffer in the dark. Embryos were then cultured for 10 hours in Na-acetate-1-C^{14} (100 mg/l, 3.6 mc/mM) in 0.1 M phosphate buffer, pH 6.0; light treatment, red, far-red, and white, 10 hours; red-far-red and far-red-red, 5 hours each treatment or 10 hours total; light intensity, red and white 222 ft-c, far-red, 210 ft-c; temperature 18°C. Embryos were from fruits grown during November, 1960.

Light Treatment	Specific Activity of Lipides	
	15-Day Embryos	17-Day Embryos
	counts/minute/mg fat	
Dark	480	5,620
White	670	11,000
Red	856	7,300
Far-red	858	3,720
Red + far-red	838	4,000
Far-red + red	817	2,470

SUMMARY

1. Light has a stimulatory effect on the synthesis of lipides by developing embryos of flax *in vitro*. The optimum intensity of white light was 100 ft-c.

2. The action spectrum of light quality *versus* specific activity shows that all wavelengths of light in the visible region stimulated the incorporation of radioacetate into lipides. The light stimulation of polyunsaturated fatty acid synthesis increased with increasing wavelengths in the visible region of the spectrum except the far-red region. Synthesis of stearic and oleic acids was enhanced by visible light of all wavelengths but was independent of the wavelength of light supplied to the culture.

3. Red light (6550 Å) stimulates and far-red light (7350 Å) inhibits synthesis of polyunsaturated fatty acids. The stimulatory effect of red light and the inhibitory effect of far-red light was observed in 17-day embryos. Other data indicate that the inhibition by far-red light was on the synthesis of polyunsaturated fatty acids. In 15-day embryos, which make very little polyunsaturated fatty acids, a red far-red effect was not observed.

Part III

Studies on the Pathways of Biosynthesis of the Fatty Acids

INTRODUCTION

It is now well accepted that fatty acid synthesis, whether in plants or animals, occurs by condensation of 2-carbon units. Glucose, sucrose, acetic acid and ethanol are various substrates that are converted to fats in most biological systems.

Various authors have described cell-free enzymatic systems that synthesized long-chain fatty acids. These systems isolated from plants and animals synthesize mainly saturated fatty acids from C_8 to C_{18}. Some systems synthesized a small amount of oleic, but none have been observed to synthesize the common polyunsaturated fatty acids, linoleic or linolenic. In spite of this, it has been thought that the mechanisms and enzymatic systems which synthesize saturated fatty acids also hold for the synthesis of both saturated and unsaturated fatty acids.

Rittenberg and Bloch (36) showed that in mice acetate was incorporated into fatty acids in an orderly fashion.

Dauben, Hoerger and Peterson (10) injected carboxy-labelled acetate into mice and isolated the fatty acids. Stepwise degradation of plamitic acid showed a "head to tail" condensation of the acetate molecules.

Stansly and Beinert (41) attempted to show that the synthesis of fatty acids was a reverse of the beta-oxidation. In this experiment, no higher fatty acids were formed. It seemed apparent that

either the conditions for synthesis of long-chain fatty acids were not met or that the synthetic process was not the reverse of the degradation process.

Brady and Gurin (7, 8) reported that long-chain fatty acids could be synthesized from acetate-1-C^{14} by a homogenate of pigeon liver. It was found that the mitochondria separated from the homogenate by centrifugation could incorporate acetate-1-C^{14} into fatty acids. Extracts of the mitochondria were also shown to be effective in the presence of Mg^{++}, DPN, ATP, and CoA.

Popjak and Tietz (34, 35, 47) showed that mouse mammary gland slices or suspensions incorporated acetate-1-C^{14} into saturated fatty acids of even chain length from C_8 to C_{18} as well as oleic acid. Krebs cycle intermediates and aerobic conditions were necessary for the synthesis.

Tietz (48) described a cell-free preparation of the fat-body of the locust that incorporated acetate into fatty acids in the presence of ATP, CoA, glutathione, Mg^{++}, TPN, malonate, α-keto-glutarate and $KHCO_3$. The major fatty acid synthesized was palmitic.

Newcomb and Stumpf (32) studied <u>in vitro</u> fatty acid synthesis in peanut cotyledon slices using acetate-1-C^{14} and glucose-U-C^{14} as the substrates.

Stumpf and Barber (46) showed that avocado mesocarp particles incorporated acetate-1-C^{14} into palmitic acid; ATP, CoA and Mn^{++} were necessary for this synthesis.

Similarly, Squires and Stumpf (40) reported a non-particulate system from avocado mesocarp particles that synthesized long-chain

fatty acids (palmitic) and required ATP, $KHCO_3$, Mg^{++}, TPNH, and CoA.

Very little is known about the biosynthesis of the unsaturated fatty acids. Research on this subject in animals and yeast has centered on: 1, attempts to isolate a fatty acid dehydrogenase; 2, in vivo synthesis and conversion of polyenoic fatty acids in animal systems. Conversion of deuterated palmitic acid to palmitoleic was reported by Stetten and Schoenheimer (44) in a feeding experiment on rats. Jacob and Pascaud (19) obtained a supernatant from rat liver that dehydrogenated palmitic acid to palmitoleic acid in the presence of hypoxanthine, the hydrogen acceptor in the reaction. In yeast, Bloomfield and Bloch (4) described a cell-free preparation which desaturated palmitic acid, and required TPNH, Mg^{++}, and molecular oxygen. Later they showed that stearic acid could be dehydrogenated to oleic acid using the same aerobic condition (5). A 10-hydroxystearic acid intermediate was postulated (23).

As a result of studies on the biosynthesis of unsaturated fatty acids in animals, the conversion of saturated to monounsaturated acids was accepted as a major pathway of fatty acid metabolism in the animal.

There was a great deal of evidence that rats synthesized polyunsaturated fatty acids of C_{20} or more when fed linoleic and linolenic acids. The studies of Mead et al. (24, 25, 26, 27, 28, 42) showed that the longer-chain (C_{20}-C_{22}) polyunsaturated fatty acids could be synthesized from linoleic and linolenic acids in the

rat liver. Mead stated that the synthesis of linoleic and linolenic acids, if it existed, was present at a very low rate (24). He suggested that there were three recognized families of polyunsaturated fatty acids rising from oleic, linoleic, and linolenic acids. The three families of acids were not readily interconvertible since double bonds were not added to the methyl ends of the molecule. The polyunsaturated fatty acids of a carbon chain length greater than C_{18} were formed by successive desaturations in divinyl methane relationship to the existing double bonds of the precursor C_{18} unsaturated fatty acids (24). That is, new double bonds were introduced into the saturated carbon chain between the existing double bonds and the carboxyl group of the acid. For example, if a double bond occured in the 9, 10 position in the carbon chain, a new double bond would be introduced in the 6, 7 position.(14).

The animal body can desaturate any unsaturated fatty acid presented to it. It is also of interest that the chain length can be increased by addition of one C_2-unit to the carboxyl end of the chain. Mead (24) suggested that linoleic and linolenic acids were not synthesized in the animal because the specific monoenoic acids were not available to it. He further stated that biohydrogenation, or a reversal of the desaturation process, was not important in animal metabolism.

In plants, increase in the iodine number of the oil of maturing seeds may be evidence for the dehydrogenation of saturated fatty acids. Painter (33), however, stated that although the iodine value of flax oil increased during maturation, the total amount of saturated

and unsaturated fatty acids increased but at different rates. This evidence suggested that the component fatty acids may be synthesized by different mechanisms.

Sims et al. (39) stated that the iodine value of the developing flax oil reached its highest value toward the mid-point of development and the presence of increasing amounts of linolenic acid suggested a desaturation process.

Experiments by Simmonds and Quackenbush (38) and Gibble and Kurtz (16) showed that in plants polyunsaturated fatty acids, especially linoleic and linolenic, are synthesized by in vitro culture.

Using C^{14}-labelled sucrose as a substrate, Simmonds and Quackenbush showed that in maturing soybeans radioactivity appeared first in oleic acid followed by the saturated acids, and then linoleic and linolenic acids, in that order. After a two-day treatment of radioactive sucrose, developing soybeans continued to assimilate the radioactive substrate into all fatty acids for five days. The total activity of oleic doubled, linoleic acid increased four-fold, and linolenic increased seven-fold. The relative percentages of the different fatty acids in the oil remained unchanged. These authors suggested that the data strongly favor conversion of oleic to the other acids, although no evidence for dehydrogenation of saturated fatty acids was obtained.

Gibble and Kurtz demonstrated that both saturated as well as unsaturated fatty acids were synthesized from Na-acetate-1-C^{14} by in vitro culture.

Hilditch (17, 18) stated that increased unsaturation in

developing seed oils is due to further synthesis of the unsaturated fatty acids, and it was unnecessary to postulate that fatty acids already formed undergo any further change. Additional evidence was presented by Hilditch in the observation that many plants grown in relatively cool climates produced seed fats of a more unsaturated nature than when grown in a warmer region. In this case, it is unlikely that a desaturation reaction would proceed more actively at a lower temperature. Based on this observation, Hilditch stated that it is extremely improbable that desaturation plays any part in the biosynthesis of unsaturated fatty acids. He suggested that the unsaturated fatty acids in seed fats were synthesized by entirely different mechanisms from that which operated in the synthesis of saturated fatty acids.

In contrast to the hypothesis of Simmonds and Quackenbush (38), who considered oleic acid to be the first fatty acid formed, Hilditch postulated that the order of appearance of the fatty acids is triene - diene - monoene (oleic in the case of the C_{18} acids). He further concluded that there is no indication that saturation of the unsaturated acids continued until the saturated (stearic) acid was produced.

METHODS AND MATERIALS

A. In vitro culture. In this study the washed flax embryos (prepared as described in Part I under section A of Methods and Materials) were placed in open 9-cm Petri dishes containing 2 pieces of Whatman #1 filter paper. Six ml of Na-acetate-1-C^{14} (10 mc/mM, 1000 mg/l) in 0.1 M phosphate buffer, pH 6.0 was added. The Petri dishes were set in a large enclosed glass dish which contained filter paper saturated with 10 per cent KOH solution. Embryos, absorbant and container were placed in a controlled-temperature chamber at 20°C. This culture was maintained in the dark for 20 to 90 minutes, depending upon the experiment performed.

At the end of the incubation period, the embryos were washed in cool phosphate buffer to remove the radioacetate solution. Some embryos were dropped immediately into boiling petroleum ether and the lipides were extracted. The remaining washed embryos were divided into groups. Each embryo group was placed in a 6.0-cm Petri dish. Two dishes contained 1.0 ml 0.1 M phosphate buffer and the other two contained 1.0 ml biotin (10 µg/l) in 0.1 M phosphate buffer. One Petri dish containing biotin and one without biotin were placed in the dark in a controlled-temperature chamber at 20°C. The remaining two Petri dishes were placed in a chamber with 200 ft-c light intensity at 20°C.

During the culture, numbers of the embryos were removed at various time intervals and dropped into boiling petroleum ether (d. 0.67-0.69) and the lipides were extracted.

B. Extraction of lipides. This is described in Part I under section F of Methods and Materials.

C. Chromatography of fatty acids. This is described in Part II under section B of Methods and Materials.

D. Analysis of natural fatty acid constituents by gas chromatography.

1. Collection and extraction of lipides. A large number of flowers were tagged on the same day. Groups of fruits of different ages were collected 18 to 30 days later. The embryos were dried at 60°C for 4 hours and macerated. The lipides were extracted into petroleum ether (d. 0.67-0.69) by refluxing for 4 hours.

2. Saponification of fatty acids. The saponification procedure is described in Part II under section B1 of Methods and Materials.

3. Esterification of fatty acids. The methyl esters were prepared with diazomethane (Appendix of Methods, Part III).

4. Gas chromatography. The ester samples were evaporated to a small volume on a hot plate and a 50 µl sample was used for analysis in the Aerograph Model A-100 gas chromatographic instrument

(Wilkens Instrument, Inc., Walnut Creek, California). The column used was a 5 ft. diethylene glycol succinate polyester column. The temperature was 200°C and the helium pressure was 20 lbs.

RESULTS AND DISCUSSION

Analysis of the fatty acid composition of developing flax embryos shows that the first fatty acids formed are saturated and oleic (Table 14). These data are in agreement with Simmonds and Quackenbush (38) who found that stearic and oleic acids were the first long-chain fatty acids synthesized in developing soybeans.

The polyunsaturated fatty acids, linoleic and linolenic, arise late in the maturation of the embryo. Eyre (12) showed that flaxseed oil increased in unsaturation during maturation of the seed.

In an _in vitro_ culture of 18-day flax embryos (Figure 4), stearic and oleic acids were also the first long-chain fatty acids synthesized, followed by linoleic and linolenic acids. This agrees with the work cited in Table 14. In these studies, stearic and oleic acids seem to be metabolically linked; linoleic and linolenic acids are similarly associated. Factors affecting the synthesis of both groups are considered in this discussion.

In 18-day embryos cultured in radioacetate _in vitro_, stearic and oleic acids were the first acids formed even though much polyunsaturated fat was present in the embryos before the start of the culture period.

If the polyunsaturated fatty acids were formed by desaturation of stearic or oleic acids, old embryos would have a greater "desaturation potential," and very little stearic and oleic acids

Table 14. Variation of fatty acid composition with embryo age as determined by gas chromatography. Embryos were from fruits grown during April, 1960.

Embryo Age	Fatty Acid			
	Palmitic	Stearic & Oleic	Linoleic	Linolenic
days		per cent		
15	tr.	tr.		
17	19	72	0	9
20	2	95	1.5	1.5
22	3	80	4	13
30	6	62	6	26
Mature	7	40	8	46

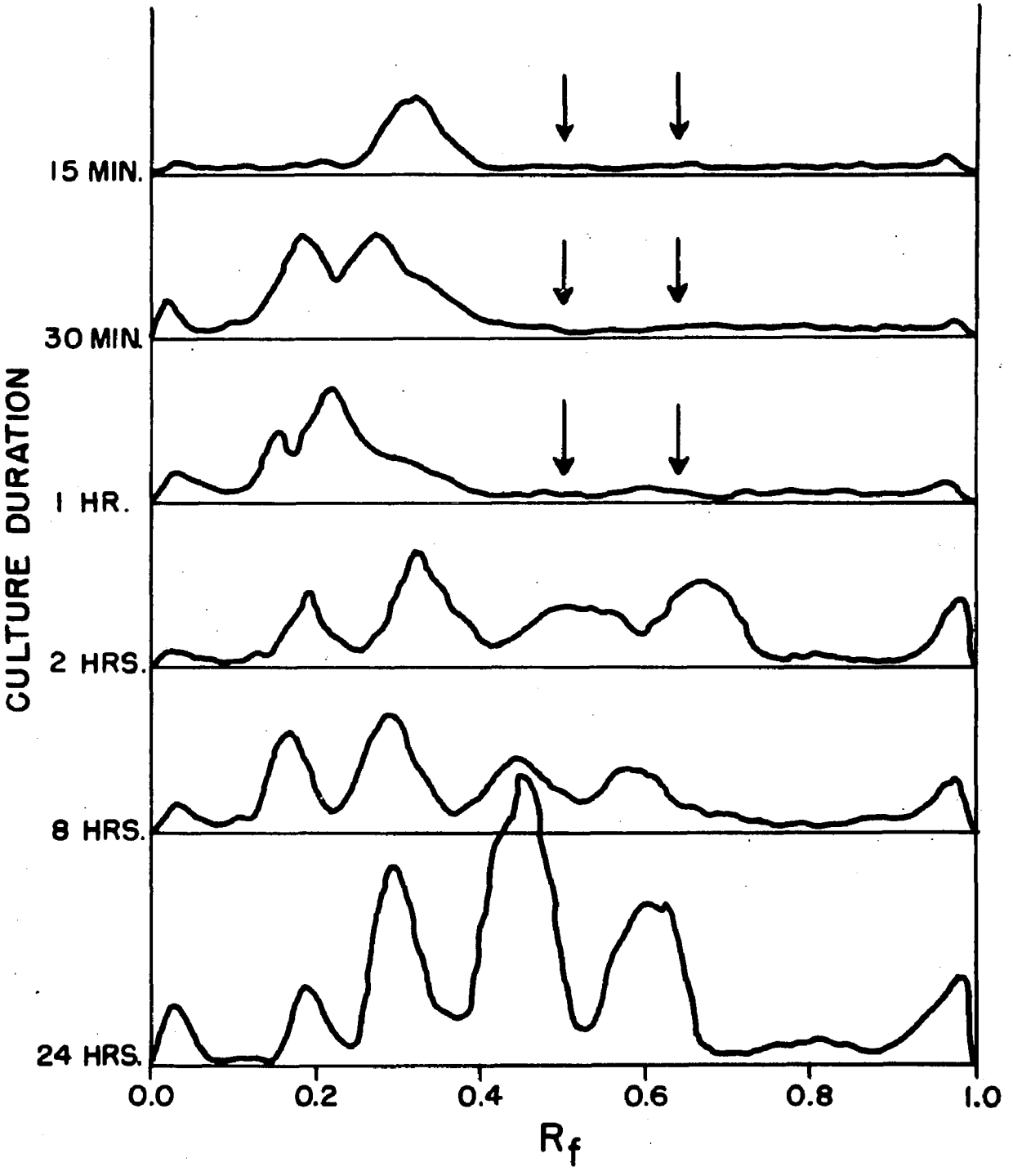

Figure 4.
Culture duration and biosynthesis of fatty acids in vitro as shown by chart recordings of strip-scanned chromatograms. Radioactive fatty acids appear as peaks. Rf's were: stearic, 0.18; oleic, 0.27; linoleic, 0.45; linolenic, 0.62. Twenty-two-day flax embryos were cultured in Na-acetate-1-C^{14} (1000 mg/l, 3.5 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 18°C. Arrows indicate areas where large amounts of non-radioactive unsaturated fatty acids were present as determined by permanganate-periodate oxidation of the strips. Embryos were from fruits grown during December, 1960.

would be present as intermediates. In such embryos which are able to make polyunsaturated fat in quantity, the appearance of appreciable amounts of stearic and oleic acids strongly indicates the presence of a separate pathway for the synthesis of the polyunsaturated fatty acids.

In developing embryos, the pathway which directs synthesis of the saturated and oleic acids appears first and is followed by the polyunsaturated pathway in old embryos. The fact that young embryos synthesize mostly saturated fatty acids, whereas old embryos synthesize both the saturated and unsaturated components, provides additional evidence.

Embryos at varying stages of maturity, in addition to their differences in synthetic capacity and fatty acid composition, synthesize fatty acids from acetate at different rates. The rate increases as a function of embryo age (Figure 5).

Tables 15 and 16 show the radioactivities of the individual fatty acids for 15 and 18-day embryos cultured in the dark. Embryos at these two ages were tagged as a single group, half were cultured at 15 days and half at 18 days. It is observed that 18-day embryos are capable of synthesizing more unsaturated fatty acids. The culture synthesis of stearic acid, however, remained virtually the same in both ages. In the culture of 18-day embryos, activity of the polyunsaturated fatty acids was increased as a function of the culture duration. In these experiments, cultured in the dark, a requirement for additional biotin could not be demonstrated.

Table 15. Fatty acid synthesis *in vitro* in fifteen-day flax embryos. Embryos were cultured in 0.1 M phosphate buffer, pH 6.0, in the dark; biotin, 10 µg/l; temperature, 20°C; after a pre-culture. Embryos were from fruits grown during March, 1961.

Additions	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/100 embryos			
Acetate-C^{14}	0	154	264	99	55
	10	281	281	148	83
Biotin	1	416	260	156	91
	3	528	330	308	220
	10	620	264	140	78
None	1	182	195	117	91
	3	360	336	228	84
	10	500	425	140	100

*One hour pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

Table 16. Fatty acid synthesis *in vitro* in eighteen-day flax embryos. These embryos are from the same group as those in Table 15, but cultured 3 days later. Embryos were cultured in 0.1 M phosphate buffer, pH 6.0, in the dark; biotin, 10 µg/l; temperature 20°C; after a pre-culture. Embryos were from fruits grown during March, 1961.

Additions	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/embryo			
Acetate-C^{14}	0	88	788	263	204
	10	1,533	2,796	1,082	4,961
Biotin	1	222	1,014	444	888
	3	188	626	501	1,534
	10	414	828	787	2,981
None	1	302	1,058	453	504
	3	164	850	458	1,145
	10	525	973	649	2,976

*One hour pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

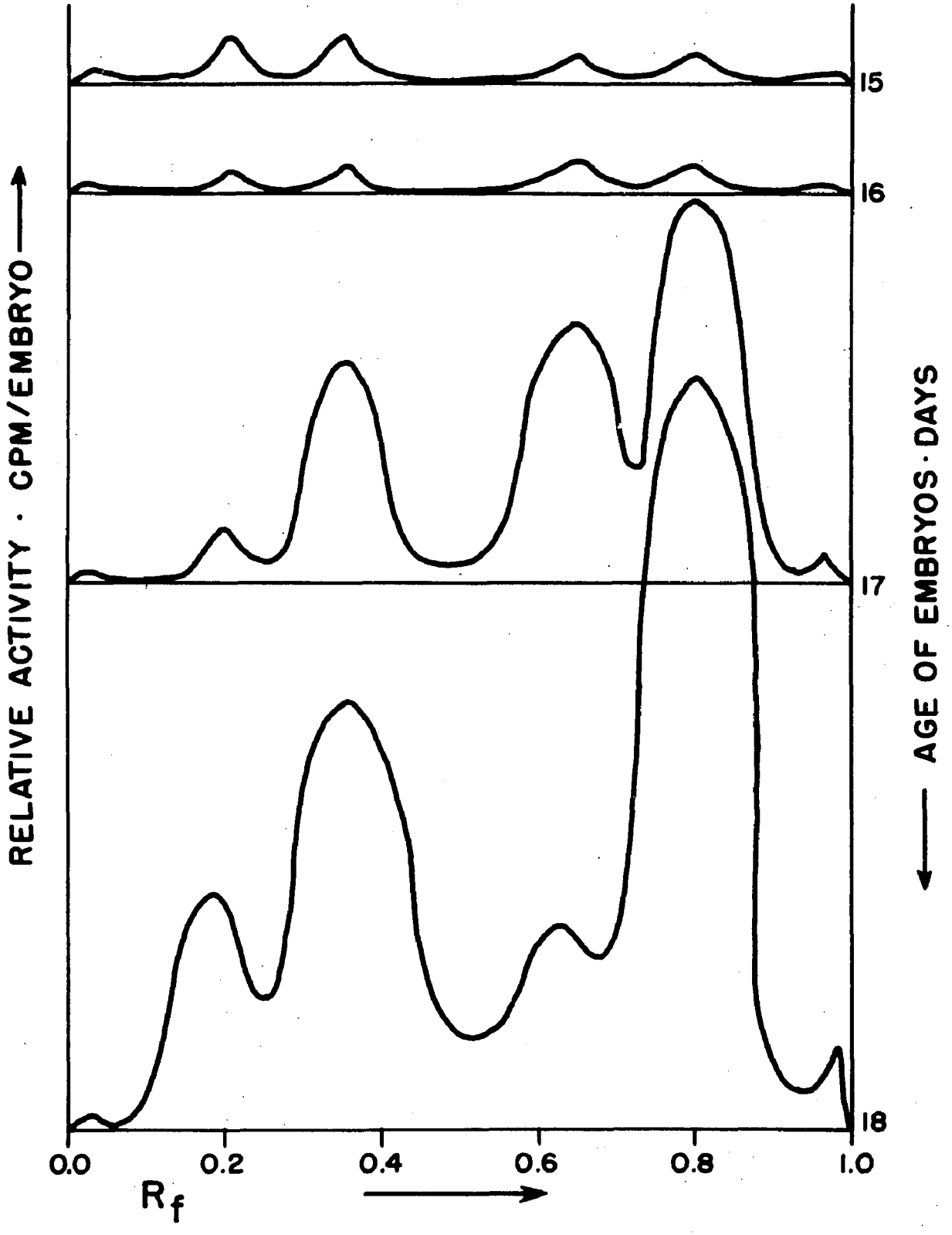

Figure 5.
Relative rates of fatty acid synthesis in maturing flax embryos. Simulated chart recordings of strip-scanned chromatograms. The fatty acids appear as peaks. Rf's were: stearic, 0.2; oleic, 0.4; linoleic, 0.6; linolenic, 0.8. Embryos were cultured for 10 hours in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; temperature, 20°C.

Table 17 shows that the maximum activity of linoleic and linolenic acids was reached after 1 to 3 hours of culture. By contrast, stearic and oleic acids showed almost no change in activity after 1 hour of culture. It is of interest to note that in the experiment of Table 17 the embryos were cultured at 17 days and weighed 0.98 mg/embryo, but in the experiment of Table 16 the embryos were cultured at 18 days and weighed only 0.75 mg/embryo. This observation shows the need of more precise environmental control during the development of fruits.

Young embryos (15-day, Table 15, and 16-day, Table 18) synthesized predominately stearic and oleic acids. Old embryos (18-day, Table 16) synthesized stearic and oleic acids early in culture, but after 10 hours linoleic and linolenic acids were the most radioactive.

It has been observed that biotin must be added simultaneously with radioacetate in culture for maximum fat synthesis. Table 19 shows that biotin, when added simultaneously to embryos cultured in radioacetate, greatly stimulated fatty acid synthesis, especially linoleic and linolenic acids. When biotin was added to embryos after a pre-culture in radioacetate (Tables 15, 16, 17 and 18), only a slight stimulation of fatty acid synthesis was observed. Biotin stimulated only when added with acetate, but light had a general enhancement on fatty acid synthesis regardless of whether it was added with radioacetate or later.

The differences of behavior of biotin and light, although both are stimulatory to fat synthesis, suggest different physiological activities for biotin and light. The sensitivity of the cultured

Table 17. Fatty acid synthesis *in vitro* in seventeen-day flax embryos. Embryos were cultured in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensity, 200 ft-c; temperature, 20°C; after a pre-culture. Embryos were from fruits grown during March, 1961.

Additions	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/embryo			
None	0	108	1,326	325	298
Dark	0.5	107	1,066	266	213
	1.0	417	2,780	973	1,668
	3.0	388	1,632	854	2,331
	8.0	298	1,428	1,666	2,499
Biotin + Dark	0.5	1,814	3,005	851	283
	1.0	1,655	3,972	1,456	794
	3.0	1,507	4,082	1,444	879
	8.0	1,036	2,486	1,036	1,036
Light	0.5	2,732	2,355	1,413	471
	1.0	1,496	1,911	1,662	747
	3.0	2,460	2,236	6,931	1,118
	8.0	2,382	2,541	5,558	1,350
Biotin + Light	0.5	586	1,172	410	527
	1.0	851	1,702	1,106	851
	3.0	551	1,047	1,212	1,047
	8.0	503	654	1,107	754

*One hour pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

**Some embryos in this treatment may have been under anaerobic conditions because of a small number of embryos which were used and some may have been covered by culture solution.

Table 18. Synthesis of long-chain fatty acids in sixteen-day flax embryos. Embryos were cultured in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensity, 200 ft-c; temperature, 20°C; after a pre-culture. Embryos were from fruits grown during March, 1961.

Additions	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/embryo			
Acetate-C^{14}	0	18	13	5	1
	8.0	41	125	157	157
Dark	0.5	32	27	14	5
	1.0	17	3	12	4
	3.0	19	20	21	6
	8.0	41	37	11	7
Biotin +	0.5	17	33	20	5
Dark	1.0	9	51	22	12
	3.0	49	44	29	11
	8.0	52	34	22	11
Light	0.5	5	15	7	3
	1.0	6	28	12	4
	3.0	33	20	17	3
	8.0	1	60	33	11
Biotin +	0.5	10	16	7	6
Light	1.0	4	26	14	9
	3.0	6	27	20	12
	8.0	82	81	59	21

*Twenty minute pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

Table 19. Effect of biotin on the synthesis of component fatty acids in sixteen-day flax embryos cultured *in vitro*. Embryos were cultured for 24 hours in Na-acetate-1-C^{14} (1000 mg/1, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; D-biotin, 10 µg/1; L-biotin, 100 µg/1; light intensity, 200 ft-c; temperature, 20°C. These embryos are the same as those in Table 20. The results shown in this table are included in Table 20. Embryos were from fruits grown during October, 1960.

	Fatty Acid			
Additions	Stearic	Oleic	Linoleic	Linolenic
	disintegrations/minute/embryo			
Light	140	870	390	200
Light + D-Biotin	128	1,050	802	1,349
Light + L-Biotin	150	1,054	1,205	903

embryos to biotin for only a limited time during early culture stages suggests that in the two synthetic pathways which are operable both require biotin in the early synthetic reactions. After a one-hour treatment in acetate, an active metabolic pool of acetate is present as evidenced by increasing fat synthesis in every case after removal of embryos from the acetate substrate (Tables 15, 16 and 18).

In young embryos, biotin has its action mainly in the synthesis of stearic and oleic acids and biotin does not stimulate the synthesis of the polyunsaturated fatty acids. Apparently the enzyme system necessary for this latter pathway may be inactive or present in low concentrations. This statement is based on the observation that young embryos synthesize mainly stearic and oleic acids (Tables 15 and 18).

At some stage of development, presumably when the enzymatic mechanisms for unsaturation are ready, biotin greatly stimulates this unsaturation (Table 19).

Table 20 gives evidence that both D and L isomers of biotin are active in stimulating synthesis of linoleic and linolenic acids. Only the D isomer, however, in conjunction with ATP, $MgCl_2$, and TPNH or DPNH increased the activity of oleic acid. One may reason then, that biotin has two roles in the synthesis of precursors of both saturated (stearic and oleic) and polyunsaturated (linoleic and linolenic) fatty acids. In the synthesis of the polyunsaturated fatty acids, both D and L isomers are active, (Table 19). Although the synthesis of polyunsaturated fatty acids was equally affected by

Table 20. Effect of biotin isomers and co-factors on synthesis of fatty acid components in sixteen-day flax embryos. These embryos are the same as those in Table 19 and the culture conditions are those of Table 19. Co-factors were: ATP, DPN, DPNH, TPNH, 1 μM; Mg^{++}, 3 μM; culture volume, 0.5 ml. Embryos were from fruits grown during October, 1960.

	Fatty Acid			
Additions	Stearic	Oleic	Linoleic	Linolenic
	disintegrations/minute/embryo			
Light	140	870	390	200
Light + D-Biotin	128	1,050	802	1,349
Light + L-Biotin	150	1,054	1,205	903
ATP, Mg^{++}, DPNH + D-Biotin	257	2,079	1,221	968
ATP, Mg^{++}, DPNH + L-Biotin	222	1,220	890	1,165
ATP, Mg^{++}, DPN + D-Biotin	217	1,130	880	1,260
ATP, Mg^{++}, DPN + L-Biotin	160	1,300	860	1,300
ATP, Mg^{++}, TPNH + D-Biotin	157	2,470	1,195	968

addition of D or L isomers, the L isomer was added in 10 times the concentration of the D isomer.

In a study of the effect of biotin analogues on 15-day flax embryos (Part I), only the D isomer of biotin was effective in stimulating lipide synthesis. This observation is in agreement with the syggestion that in young embryos only the D specific saturated fatty acid pathway would be operable.

Table 20 shows that a mixture of ATP, $MgCl_2$ and DPNH or TPNH and D-biotin is effective in stimulating oleic acid synthesis, in agreement with published data (40). It is thought that the action of light, at least in the synthesis of stearic and oleic acids, is to supply energy for the reactions through photosynthetic phosphorylation. The role of light in the synthesis of unsaturated fatty acids is far more subtle, since addition of co-factors had no effect on increasing the activity of the polyunsaturated fatty acids.

Light stimulates the synthesis of all fatty acid components, especially linoleic and linolenic acids, when embryos were cultured aerobically (Table 21). Embryos cultured anaerobically under nitrogen atmosphere in light without biotin synthesized stearic and oleic acids in great quantities, but linoleic and linolenic acids showed very little activity. This indicates a dependence of the polyunsaturated pathway upon an aerobic condition.

It has been observed that biotin can function either in light or in the dark to enhance synthesis of polyunsaturated fatty acids

Table 21. Fatty acid synthesis *in vitro* in eighteen-day flax embryos cultured under air and nitrogen. Embryos were cultured for 8 hours under air and nitrogen in 0.1 M phosphate buffer, pH 6.0; biotin, 10 µg/l; light intensity, 200 ft-c; temperature, 20°C; after a pre-culture. Embryos were from fruits grown during April, 1961.

Additions	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/embryo			
Air					
Acetate-C^{14} + Dark	0	36	54	18	18
	8	131	241	544	469
Dark	8	25	54	54	217
Biotin + Dark	8	100	100	212	357
Light	8	96	205	370	498
Biotin + Light	8	45	134	112	391
Nitrogen**					
Dark	8	95	110	61	35
Biotin + Dark	8	108	492	184	768
Light	8	724	1,810	314	290
Biotin*** + Light	8	43	109	22	22

*One hour pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

**Culture was depleted of nitrogen supply after 3 hours.

***The non-saponifiable material in this treatment was 4 times more radioactive than the non-saponifiable material in the corresponding aerobic culture.

in old embryos (Table 18). Although light is stimulatory, cultures maintained in the dark can also synthesize polyunsaturated fatty acids. In most cases, however, synthesis in light is greater. Table 21 shows that under anaerobic (nitrogen) conditions, synthesis of the polyunsaturated fatty acids in the dark is increased when biotin is added. The action of biotin in the polyunsaturated pathway seems to have a dual nature. One action is to enhance synthesis in the dark and the other action is to enhance synthesis in the light. The dark pathway of linolenic acid synthesis is stimulated by anaerobic culture conditions, whereas the light pathway is inhibited by this anaerobic condition. Anaerobiosis stimulates the synthesis of the stearic and oleic acids under most conditions studied.

The requirement of an aerobic system for the synthesis of oleic acid was reported in yeast by Bloomfield and Bloch (5) and in mouse mammary tissue by Popjak and Tietz (34, 35). The synthesis of polyunsaturated fatty acids is greatly inhibited under anaerobic conditions in light (Table 22 and Figure 6). This agrees with the results in Table 21. It can be shown that the activity of the polyunsaturated fatty acids increased with culture time under both aerobic conditions of air and oxygen. Under nitrogen, however, synthesis of the polyunsaturated fatty acids was greatly inhibited.

Whether conversions or hydrogenation-dehydrogenation reactions occur between linolenic and linoleic acids, between stearic and

Table 22. Effects of air, oxygen and nitrogen on the *in vitro* synthesis of long-chain fatty acids in eighteen-day flax embryos. Embryos were cultured under air, oxygen, or nitrogen in 0.1 M phosphate buffer, pH 6.0; light intensity, 200 ft-c; temperature, 20°C; after a pre-culture. These data are shown graphically in Figure 6. Embryos were from fruits grown during June, 1961.

Culture Gas	Time After Pre-Culture*	Fatty Acid			
		Stearic	Oleic	Linoleic	Linolenic
	hours	disintegrations/minute/embryo			
Air	0	435	820	507	435
	1	3,816	13,954	261	1,635
	3	2,517	20,697	8,391	10,349
	8	2,238	12,307	8,950	15,943
Oxygen	1	1,353	7,079	1,770	1,249
	3	6,350	20,493	10,967	3,064
	8	2,163	11,256	6,783	17,318
Nitrogen**	1	1,083	9,206	541	270
	3	3,202	27,273	1,143	457
	8	26,168	42,552	3,868	2,023

*One hour pre-culture in the dark in Na-acetate-1-C^{14} (1000 mg/l, 10 mc/mM) in 0.1 M phosphate buffer, pH 6.0; temperature, 20°C.

**Culture was depleted of nitrogen supply after 3 hours culture time.

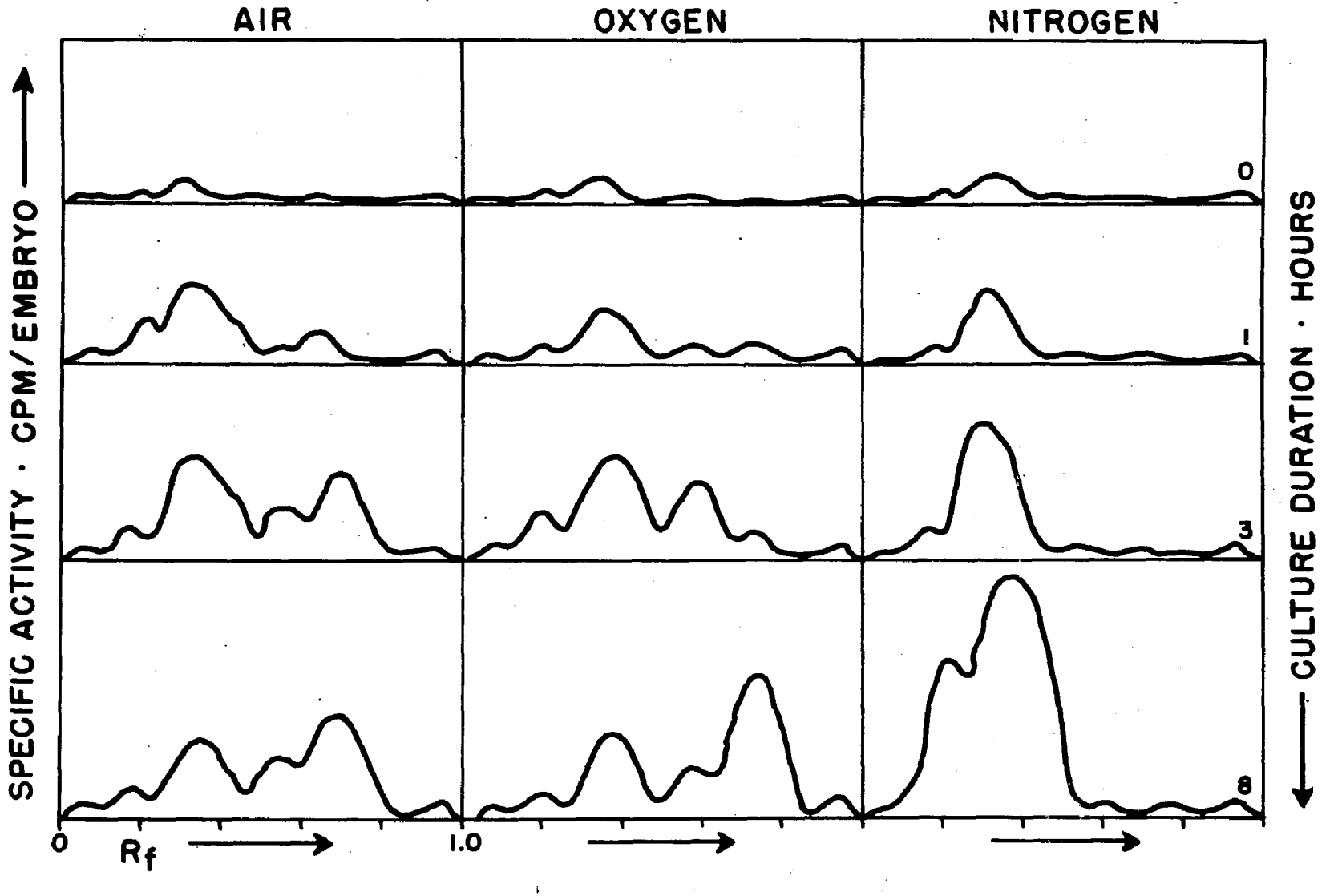

Figure 6.
Synthesis of fatty acids in vitro in air, oxygen, and nitrogen as shown by chart recordings of strip-scanned chromatograms. The radioactive fatty acids appear as peaks. Rf's were: stearic, 0.20; oleic, 0.40; linoleic, 0.60; linolenic, 0.75. This figure is a graphic representation of the data presented in Table 22.

oleic acids, or between these two groups of acids is not known at this time.

SUMMARY

1. Fatty acid extracts of developing flax embryos were analyzed by gas chromatography. Results show that young embryos synthesize mainly saturated and oleic acids. Old embryos synthesize linoleic and linolenic acids in addition to saturated and oleic acids.

2. Cultures of flax embryos *in vitro* showed that acetate-1-C^{14} was incorporated into stearic, oleic, linoleic and linolenic acids. In these experiments, stearic and oleic acids were synthesized during the shortest culture times and linoleic and linolenic acids appeared later. The order of synthesis of the fatty acid constituents in flax embryos is the same *in vivo* and *in vitro*.

3. There is evidence that in the flax embryo stearic and oleic acids are synthesized by one metabolic pathway and linoleic and linolenic acids are synthesized by another. The pathway for saturated and oleic acids arises early in the development of the embryo; the polyunsaturated route develops mainly in old embryos.

4. Biotin appears to be necessary in the early synthetic reactions of both metabolic pathways. Both the D and L isomers of biotin can stimulate the synthesis of the polyunsaturated fatty acids.

5. Light has a stimulatory effect on the synthesis of all fatty acid components. Although light is stimulatory, it seems to be

unnecessary for the synthesis of polyunsaturated fatty acids. This stimulation of polyunsaturation in the dark occurs under both aerobic and anaerobic culture conditions. Anaerobiosis stimulates synthesis of the polyunsaturated fatty acids in the dark in the presence of biotin.

GENERAL SUMMARY

1. Biotin is a constituent of flax plants and is an important factor in the synthesis of fatty acids in the flax embryos. The biotin content of the flax embryo rapidly increases during maturation. This rapid increase of biotin coincides with the physiological age at which fatty acid synthesis of the embryo is stimulated by addition of biotin to cultured embryos.

2. Excised flax embryos cultured in an acetate buffer do not synthesize biotin. It is thought that the synthesis of biotin is extra-embryonic.

3. Radioacetate is incorporated into the fatty acids of developing flax embryos.

4. In the synthesis of stearic and oleic acids, light was stimulatory. Synthesis of linoleic and linolenic acids increased with the wavelength of light supplied to the culture. Far-red light, however, inhibited the synthesis of polyunsaturated fatty acids.

5. In the development of the flax seed, young embryos synthesize mainly stearic and oleic acids, but old embryos synthesize linoleic and linolenic acids in addition to stearic and oleic acids.

6. Results of *in vitro* and *in vivo* studies show that the synthesis of stearic and oleic acids is followed by synthesis of linoleic and linolenic acids in a general pattern by which the long-chain fatty acids are formed in sequence.

7. It is concluded that two metabolic pathways, or enzyme systems, direct the synthesis of long-chain fatty acids that occur in the flax embryo. By one pathway stearic and oleic acids are synthesized, and linoleic and linolenic acids are synthesized by another. Biotin appears to be involved in the early reactions of both metabolic pathways.

8. Experiments indicate that there is a dark and a light pathway in the synthesis of polyunsaturated fatty acids. The light pathway of polyunsaturated fatty acid synthesis is inhibited by anaerobic culture conditions. The dark pathway of synthesis of polyunsaturated fatty acid is inhibited by either aerobic or anaerobic conditions.

9. Under dark anaerobic culture conditions, biotin stimulates the synthesis of oleic and linoleic acids.

10. Figure 7 summarizes the data graphically.

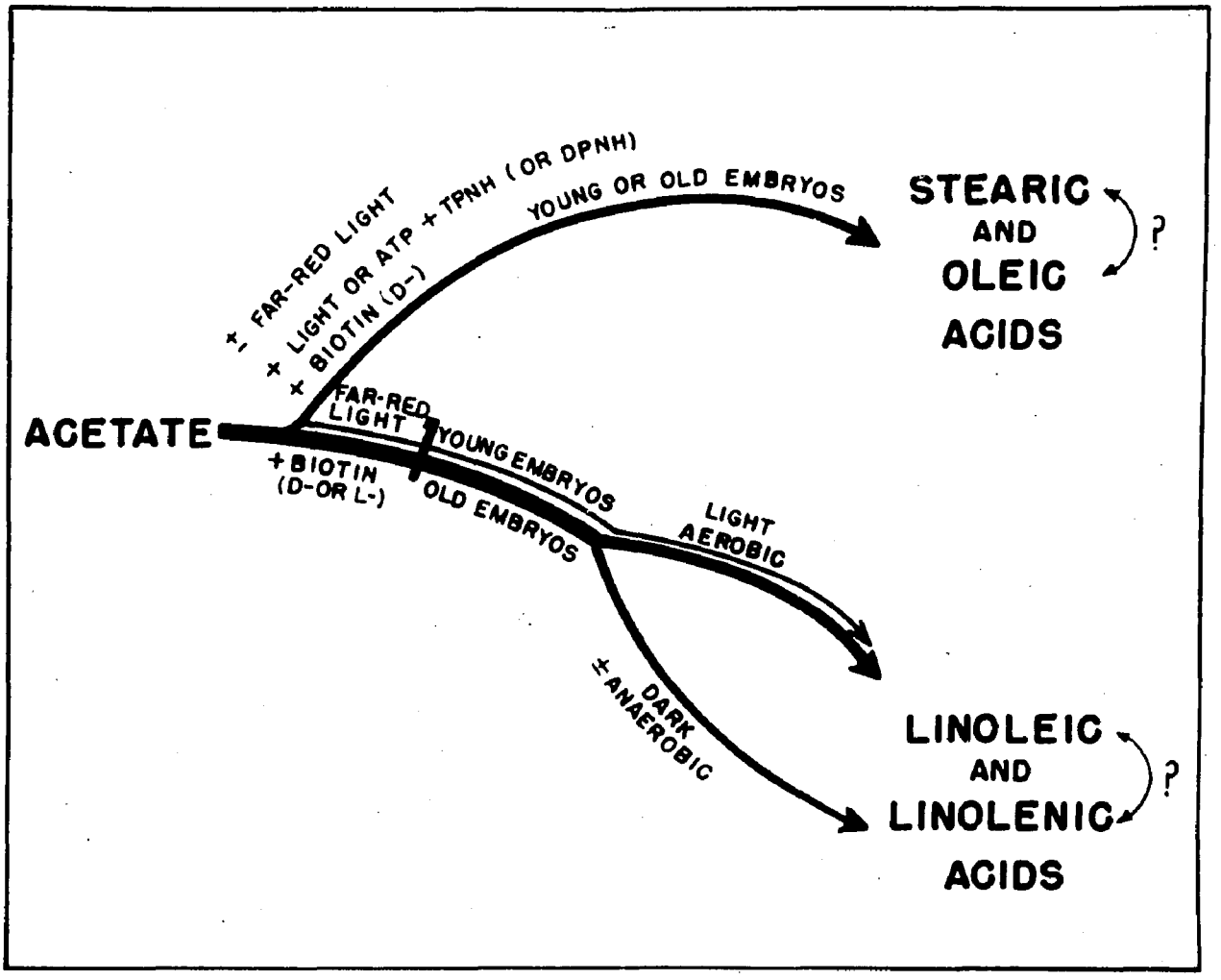

Figure 7.
Schematic representation of the pathways of synthesis of the component fatty acids in flax embryos. Thickness of the arrows designate relative amounts of fatty acids formed. Stimulation of synthesis is shown by (+) sign, and (±) means no effect. Lines crossing arrows indicate inhibition of the pathway.

APPENDIX OF METHODS

Part I

Biotin Assay

A modification of the method of Wright and Skeggs (58) was used for assay of biotin. The procedure followed was a modification of that outlined in the Methods of Vitamin Assay (29). Specific modifications of the method are described in Part I under section B of Methods and Materials.

Extraction of biotin.

The embryos to be analyzed are dried at 60°C for 4 hours. The dried material is weighed, placed in a 50-ml erlenmeyer flask, and 25 ml of 2 N sulfuric acid is added. The flask is plugged with cotton and autoclaved for 90 minutes at 15 lbs. pressure.

When cool, the solution is filtered into a separatory funnel of 60-ml capacity, and shaken with 15 ml of petroleum ether (d. 0.67-0.69) to remove oleic acid and other acids that might interfere with the assay.

The aqueous layer is placed in a 100-ml volumetric flask and brought to volume. A 10-ml aliquot is placed in a 100-ml beaker, and 10 ml of distilled water is added. The pH of this solution is adjusted to 6.8 with 1 per cent aqueous NaOH. The solution is transferred quantitatively to a 50-ml volumetric flask and stored in the refrigerator until used.

Stock culture of Lactobacillus arabinosus 17-5.

Bacto Micro Assay Culture Agar (Difco) is used for maintaining the stock cultures. The L. arabinosus is transferred by means of stabs with an inoculating loop, and then incubated for 24 hours at 37°C. Stock cultures of L. arabinosus are made fresh before each assay.

Inoculum medium for growth of Lactobacillus arabinosus.

A 1:500 dilution of a biotin stock solution is prepared by adding 1 ml of biotin stock solution (25.0 mg D-biotin in 500 ml 50 per cent aqueous ethanol) to 500 ml Bacto Biotin Assay Medium (Difco). This was the inoculum medium used.

Five ml of the inoculum medium and 5 ml of distilled water are added to each screw-cap culture tube. The tubes are autoclaved at 15 lbs. for 15 minutes, cooled, and inoculated from the stock culture using a platinum wire inoculating loop. Only fresh stock cultures no older than 24 hours post-incubation should be used. Attenuation and poor growth result from the use of older stock cultures. The inoculum medium is incubated for 24 hours at 37°C and used immediately for inoculation of standard and assay tubes.

Preparation of standard and assay tubes.

1. Working biotin standard: Dilute 5 ml of the biotin stock solution (25.0 mg D-biotin per 500 ml 50 per cent aqueous ethanol) to 250 ml with 50 per cent ethanol. Dilute 5 ml of this solution to 500 ml with 50 per cent ethanol. Prepare the working biotin standard

before every assay by diluting 5 ml of the latter solution to 250 ml with water. This working biotin standard contains 0.2 mμg biotin per ml.

2. Standard and assay media: Five ml of the Bacto Biotin Assay Medium (Difco) is added to all culture tubes (16 mm by 150 mm screw-cap culture tubes) and 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 4.0 ml, and 5.0 ml of the working biotin standard are added separately to prepare the standard tubes. The assay tubes are prepared by adding 0.5 ml or 1.0 ml of the biotin extract to 5 ml of the Bacto Biotin Assay Medium. Standard and assay tubes are made to a total volume of 10 ml with distilled water. All tubes are sterilized for 15 minutes at 15 lbs. pressure in the autoclave.

Inoculation of standard and assay tubes.

After the tubes are removed from the autoclave and cooled, they are ready for inoculation. The inoculum medium is poured into a sterile 15-ml centrifuge tube and centrifuged for 5 minutes. The supernatant is discarded and the packed cells are re-suspended in 8 ml of sterile isotonic saline (0.9 per cent NaCl in water). The cells are re-centrifuged, and the supernatant discarded. The cells are re-suspended in 8 ml of saline and poured into a sterile syringe fitted with a #25 needle. As drops begin to fall from the needle, two drops are placed in each of the standard and assay tubes. All tubes are flamed, capped and placed in a water-bath and incubated for 72 hours at 37°C.

Titration.

After the incubation period, the lactic acid formed is titrated with 0.1 N NaOH to pH 6.8, as measured by a Beckman pH meter. Cultures are placed in a beaker containing the electrodes and a magnetic stirrer. The sodium hydroxide is dispensed from an automatic burette.[1]

Calculation.

Plotting ml sodium hydroxide versus biotin concentration of the standard tubes gives a standard biotin curve. From this curve the amount of biotin in the assay tubes can be determined by the amount of sodium hydroxide used in titration.

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[1]The author is indebted to Dr. Mitchell G. Vavich for the suggestion of this titration method.

APPENDIX OF METHODS

Part II

Chromatography of fatty acids (31)

Preparation of the paper strips.

The paper strips (about 200 cm x 2.5 cm) are washed by a modified method of Ashley and Westphal (3). Each paper strip is rolled up and placed over a polyethylene sieve and washed by immersion in the following solvents in sequence:

1 liter of 10 per cent HCl
5 separate 1-liter portions of distilled water
2 separate 1-liter volumes of 95 per cent ethanol
3 separate 1-liter volumes of acetone
1 liter of petroleum ether (d. 0.67-0.69)

The washed strips are air-dried and stored until used.

Impregnation of the paper strips.

Paraffin oil (C-5572, Central Scientific Co.) is used as the stationary phase. Solutions of paraffin oil in petroleum ether (d. 0.67-0.69) are used to impregnate the washed paper strips. Concentrations of paraffin oil are 75 and 90 g/l. The washed paper strips are impregnated by immersing them in the paraffin oil solution. The strips are then air-dried and used immediately.

The prepared paper strips are placed on Kurtz-Miramon chromatographic racks (California Laboratories, Inc., Berkeley, California).

Spotting and developing the chromatograms.

The reference and unknown mixtures of fatty acids are dissolved in petroleum ether (d. 0.67-0.69) and spotted onto the starting line of the paper strips by means of micropipettes. The strips are air-dried and placed in the chromatographic chamber. A beaker of the developing solvent (90 per cent acetic acid saturated with paraffin oil) is set is the chamber to saturate the strips and chamber atmosphere. The developing solvent is prepared by shaking 500 ml of 90 per cent acetic acid with 50 ml of paraffin oil in a separatory funnel. The acidic layer is removed and used.

After the chamber and the strips have been saturated, the developing solvent is poured into the bottom of the chromatography chamber and the solvent is allowed to ascend the strips for 30 hours.

The strips are removed from the chamber, the solvent front is marked with a pencil, and the strips are allowed to dry at room temperature. The solvent is most easily detected under ultraviolet light.

The strips containing the reference fatty acids are placed in a shallow tray of 10 per cent aqueous lead acetate for 2 minutes and then washed with distilled water (usually 3 times) until the rinse water shows no trace of lead ions when tested with dilute HCl. The strips are air-dried and then oven-dried for 15 minutes at 100°C.

The strips are placed in an H_2S chamber for 15 to 20 seconds. The fatty acids appear as light or dark brown spots. The Rf of the reference fatty acids are determined. Table 23 compares the Rf

values of the reference fatty acids with 2 concentrations of paraffin oil as the stationary phase.

Table 23. Comparison of Rf values of reference fatty acids with concentration of paraffin oil as the stationary phase.

Fatty Acid	Rf Paraffin Oil Concentration 75 g/l	 90 g/l
Stearic	0.20 ± 0.02*	0.18 ± 0.02
Oleic	0.41 ± 0.05	0.28 ± 0.04
Palmitic	0.45 ± 0.03	0.30 ± 0.04
Linoleic	0.62 ± 0.03	0.45 ± 0.03
Linolenic	0.76 ± 0.04	0.62 ± 0.04

*Standard deviation, n = 10.

APPENDIX OF METHODS

Part III

Procedure for esterification by diazomethane

Methylation by conventional methods usually results in some shifting of the double bonds in the unsaturated fatty acids. To prevent this, methylation by diazomethane has been reported to be more satisfactory.

Preparation of p-toluenesulfonylmethylnitrosoamide (11).

Fifteen g of p-toluenesulfonyl chloride is divided into three portions as follows: 9.0 g, 4.5 g, and 1.5 g.

The 9.0 g portion of p-toluenesulfonyl chloride is added, with swirling for 5 minutes, to a 10 ml volume of aqueous 33 per cent methylamine in a 100 ml round-bottom flask. The mixture is heated to 80°C to maintain the sulfonylmethylamide (m.p. 78°C) in the liquid state.

After the 9.0-g portion of the p-toluenesulfonyl chloride is added, the mixture is shaken vigorously. The reaction flask is cooled with water to prevent boiling and loss of methylamine.

When the mixture is acid to litmus, 2.3 ml of 50 per cent NaOH is added carefully with swirling, followed immediately with gradual addition of the 4.5 g portion of the p-toluenesulfonyl chloride. When the mixture again becomes acid, 1.3 ml of NaOH is

added followed by the final addition of the 1.5 g portion of the p-toluenesulfonyl chloride. When the mixture becomes acidic, more NaOH is added dropwise until the mixture becomes alkaline.

The wall of the reaction flask is rinsed with a little water and the reaction is completed by stirring the mixture while it is heated on the steam bath for 15 minutes.

The hot mixture is poured into 70 ml of glacial acetic acid in a 250-ml round-bottom flask. The reaction flask is rinsed with 12 ml of glacial acetic acid and added to the round-bottom flask.

The solution is cooled in an ice bath to 5°C and stirred mechanically as a solution of 58.5 g of $NaNO_2$ in 11.8 ml of water is added dropwise from a dropping funnel. The $NaNO_2$ is added for 45 minutes during which time the temperature of the reaction is kept below 10°C. After the addition is completed, the stirring is continued for an additional 15 minutes.

Fifty ml of water is added to the mixture and the precipitate is separated by suction filtration and washed with 25 ml cold water.

The product was transferred to a beaker and stirred with 25 ml of cold water and again filtered. This process was repeated until there was no odor of acetic acid.

The product was vacuum dried over H_2SO_4 and stored in a dark bottle.

Recrystallization of p-toluenesulfonylmethylnitrosoamide.

Ten g of the reagent is dissolved in 10 ml of boiling ether and an equal volume of petroleum ether (d. 0.64) is added; the mixture is cooled overnight in a refrigerator. The crystallized reagent is collected by suction filtration and washed with cold petroleum ether.

Preparation of diazomethane and esterification of the fatty acids.

Diazomethane is generated from p-toluenesulfonylmethylnitrosoamide reagent by the addition of alcoholic KOH. The reagent is dissolved in 50 ml of diethyl ether and transferred to the reaction flask; an addional 10 ml is used to rinse the transferring beaker. The ether solution is cooled between 0° and 5°C in an ice bath. One and one-half g of KOH is added dropwise from a dropping funnel. When all the alcoholic KOH is added, the dropping funnel is removed and replaced with a glass stopper to prevent any refluxing of the diazomethane-ether into the dropping funnel. The ice bath is removed and the diazomethane-ether solution is refluxed at 35°C. When most of the diazomethane-ether solution has distilled over, the flask containing the diazomethane-ether is removed. This solution is added to the fatty acid samples in quantities of 3 moles reagent per mole of fatty acid.

In order to promote a more complete methylation of the samples, and at the same time remove the excess diazomethane, the ether-ester solution is left standing at room temperature overnight. Any remaining ether is then evaporated over a low hot plate.

To remove unreacted fatty acids, if any, the ester residue is dissolved in 20 ml of diethyl ether and shaken in a separatory funnel with an equal volume of 10 per cent aqueous $NaHCO_3$. The aqueous layer is removed and the ether layer is transferred to a 50-ml erlenmeyer flask and dried over 10 g anhydrous $MgSO_4$ for 4 hours and then filtered. The ether is evaporated and the esters are dissolved in 10 ml of n-heptane, a better solvent for gas chromatography.

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