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UNIVERSITY OF LOUISVILLE

Studies on Synthesis by Enzymatic Catalysis

A Dissertation

Submitted to the Faculty

Of the Graduate School of the University of Louisville

In Partial Fulfillment of the

Requirements for the Degree

of Master of Science

Department of Biochemistry

By

Charles Henry Wabnitz

Year

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NAME OF STUDENT: Charles H. Wabnitz

TITLE OF THESIS: STUDIES ON SYNTHESIS
BY ENZYMATIC CATALYSIS

APPROVED BY READING COMMITTEE COMPOSED OF THE
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TABLE OF CONTENTS

	Page
I. INTRODUCTION.....	1
A. Reversibility of Enzyme Action.....	2
1. Theoretical.....	2
2. Historical.....	2
3. Discussion.....	4
B. Purpose of This Research.....	6
II. ENZYMATIC SYNTHESIS OF GLYCERIDES FROM GLYCEROL AND LINSEED OIL FATTY ACIDS.....	7
A. Objectives.....	8
B. Problems.....	8
C. Literature.....	9
D. Methods.....	12
E. Experimental.....	13
F. Conclusions.....	25
G. Summary.....	26
III. ENZYMATIC SYNTHESIS OF "SUGAR ESTERS" FROM DEXTROSE AND LINSEED OIL FATTY ACIDS.....	31
A. Objectives.....	32
B. Problems.....	32
C. Experimental.....	33
D. Summary.....	50
E. Conclusions.....	50
IV. GENERAL SUMMARY AND CONCLUSIONS.....	59
V. BIBLIOGRAPHY.....	63

INTRODUCTION

The demonstration of the reversibility of enzyme action is not altogether new. Although not all enzymes have been found as yet to possess reversible action, neither is the field limited to the synthesis of merely a few compounds.

Enzymes are usually thought of as being catalysts and if they are to be classified as such one might surmise that they should possibly be able to catalyze a reaction in either direction. That is, we might expect to find enzyme reactions reversible if the substrate conditions are properly controlled. Further considerations of theoretical bearing will be given in appropriate places throughout this work.

As far back as 1892 Tammann (1) and in 1898 Van't Hoff (2) mentioned the possibility of a reversal of enzyme action. Since that time it has been repeatedly demonstrated by many workers that, as Tauber (3) so aptly states, "Under certain definite conditions reactions for some enzymes may be reversed."

Croft Hill (4) in 1898 claimed success in synthesizing maltose by digesting a forty per cent glucose substrate with yeast maltase at thirty degrees Centigrade. This was perhaps the first demonstration of synthesis by enzymatic catalysis.

Later, Emerling (5) in 1910 showed that the substance thus synthesized according to Croft Hill's method was not maltose but iso-maltose. Other workers have repeated similar experiments with these sugars with positive results.

Bourquelot and Bridel (6) were able to synthesize the glucoside salicin using the enzyme emulsin as catalyst. Other glucosides have been synthesized by them and by other workers

using emulsin and bottom fractions of brewers yeast as sources of enzymatic catalysts.

A ~~compound~~ called "plastein" has allegedly been formed from peptones by several workers. Of special note in this field is the work of Salter and Pearson (7) who were able to produce a plastein from the action of pepsin on a concentrated thyroid gland digest. They found that this plastein had a marked effect in relieving symptoms of thyroid deficiency whereas the original digest was ineffective.

Wastneys and Borsook (8) resynthesized thirty-nine percent of a peptic digest of egg albumin. Other protein syntheses have recently been reported (9).

In 1923 Kay demonstrated synthesis of urea by action of urease on ammonium carbonate and carbamate mixture.

Many workers have demonstrated synthesis of various esters and glycerides through the agency of enzymatic catalysis. A review of the work done in this field will be given subsequently in this paper when more specific details concerning this particular field are discussed.

Just recently Hassid, Dourdorff and Barker (11) (12) obtained synthetic, crystalline sucrose from glucose-1-phosphate and fructose through the enzymatic catalysis of sucrose phosphorylase from *Pseudomonas Saccharophila*.

The enzymatic synthesis of starch has also been recently announced. (13) (14)

It has therefore been demonstrated by many workers in many different cases that various chemical compounds, many of which

are of biochemical interest, can be synthesized in vitro by the apparent catalytic action of enzymes.

It must also be noted however that even though enzymes can catalyze synthesis, as Sumner and Somers (14) point out, "this does not mean that every substance which is synthesized in plants and animals is built up by the same enzyme which is able to break it down."

For example, urease hydrolyzes urea but urea is not necessarily synthesized in animals by urease but probably by the action of arginase upon arginine. Nevertheless, as has already been pointed out, urea can be synthesized by urease in vitro. In a similar way, starch is hydrolyzed to maltose by amylase but is synthesized apparently by dephosphorylation of glucose-1-phosphate by phosphorylase.

So, even though some enzymes may catalyze synthesis in the animal body, and even though we can demonstrate in vitro the reversibility of certain enzymes, it does not necessarily follow that synthesis must always be catalyzed by that same particular enzyme which will also hydrolyze the compound to be formed.

However, neither does this rule out the possibility that in at least some cases the same enzyme may both hydrolyze and also synthesize in the plant and animal according to the nature of the substrate and that possibly the concentrations of certain governing factors in the substrate may change so as to favor hydrolysis under one set of conditions and synthesis under another set of conditions. Of course there exists the possibility also

that a separate enzyme is present for specific action under each set of conditions instead of one enzyme being capable of either hydrolysis or synthesis.

It has been found that in the synthesis of oil in such oil-bearing seeds as castor beans, that as the beans mature and as the oil is formed in the seed, the lipase content increases and the water content decreases. In germination on the other hand, the water content is said to increase and the lipase hydrolyzes the oil. It may be inferred from this that possibly the same lipase is capable of either synthesizing or hydrolyzing the oil according to the substrate available. At least this has apparently been the general assumption of workers in this field.

It has also been suggested, although never adequately proven nor disproven, that lipase may play an important role in the resynthesis of fat in the epithelial lining of the intestine or even in the Golgi apparatus which is apparently the site of fat resynthesis.

Many interesting examples of enzymatic synthesis have been reported in the literature other than the comparative few listed here. This field of synthesis as brought about by the agency of enzymatic catalysis presents an enormous array of research possibilities the results of which may someday throw much-needed light upon the role of enzymes in the complex and intricate metabolism and catabolism in both health and disease of both plants and animals. There also exist many possibilities for the employment of enzymatic catalysis to promote synthesis on a large scale in various industries where at present much time and a large quantity of energy of one kind or another is required.

It must be pointed out however that enzyme work is not easily carried out nor are the results of enzymatic experimentation always readily interpreted or evaluated. This is due in part to the intangible nature of enzymes themselves and in part to the many entities and groups of entities which definitely effect them.

Therefore, for the most part, in enzyme work of the nature herein reported, methods of pure exploration must of necessity be employed and results must be interpreted cautiously and any conclusions drawn should be accepted with reservations. It is quite difficult in many cases to adequately evaluate work of this nature, but the task is somewhat easier if an open mind is kept concerning the many difficulties involved and if the pioneering aspect of such research attempts is properly considered.

In brief, the purpose of the investigation herein reported has been to seek further evidence concerning the possibility of synthesizing glycerides in vitro through the reversible, catalytic action of lipase in order to throw some light upon the possible mechanism concerned in the enzymatic synthesis of oils and fats in plants and animals as well as the possibility of synthesizing tailor-made glyceride or esters of fatty acids and polyhydric alcohols in the laboratory.

The following pages of this work represent studies and experimental results concerning these problems as carried on over a period of four years in the research laboratory of the Department of Biochemistry at the University of Louisville School of Medicine.

ENZYMATIC SYNTHESIS OF GLYCERIDES

FROM GLYCEROL AND LINSEED OIL FATTY ACIDS

The first goal set to be achieved in this work was to effect a decrease in the acid number of a mixture containing linseed oil fatty acids and glycerol through the catalytic action of lipase.

It has been assumed, relative to results obtained by other investigators in work of this nature that oil synthesis occurs in proportion to the amount of decrease in free fatty acid content of the substrate used.

Since so many factors may effect the results in work of this kind, each problem must of necessity be solved more or less as it appears in relation to the whole before the main objective is attained and each individual problem generally bears some relationship to all of the other problems involved.

Generally speaking however the problems resolved themselves into two groups. In the first place, there were problems concerning the enzyme or catalyst used, such as the best source of the enzyme, methods of extraction and activation, and the proper use of it in order to obtain maximum results. In the second place, there were problems concerning the substrate, such as the necessity to ascertain the proper ratio of glycerol to fatty acids required to obtain optimum synthesis, the effects of various concentrations of water with respect to different glycerol-fatty acid ratios and to different enzyme preparations, as well as effects of pH, temperature and other factors.

Many workers have been able to synthesize various esters through the apparent catalytic agency of lipase from an animal source.

For example, in 1900 Kastle and Loevenhart (15) synthesized ethyl butyrate from ethyl alcohol and isobutyric acid with pancreatic lipase as catalyst.

Pottevin (16) in 1904 synthesized methyl oleate and later (1905) he also obtained esterification using pancreatic lipase with oleic acid and ethyl, methyl, propyl, isopropyl and other alcohols (17).

About this same time, Bodenstein and Deitz (18) (19) esterified butyric acid and isoamyl alcohol using pancreatic lipase.

Glycerol esters have also been synthesized by several workers using pancreatic lipase.

For example, Hanriot (20) produced monobutyrim by the catalytic action of pancreatic lipase on butyric acid and glycerol, and Pottevin (21) produced mono and di oleins with pancreatic lipase. Triolein was produced by Taylor (22) using pancreatic powder, glycerol and oleic acid.

Hamsik (23) found that powder prepared from the mucous membrane of the small intestine of horses, sheep and hogs caused synthesis with oleic acid and glycerol; (24) pancreatic lipase of the pig caused fat synthesis with palmitic and stearic acids and glycerol but similar preparation from cattle and dogs did not do this.

Still other workers have used lipase from a vegetable source, namely from castor beans, to effect synthesis of glycerides.

It was first shown by J. Reynolds Green in 1889 (25) that germinating seeds of the castor oil plant contain an enzyme

capable of hydrolyzing castor oil. An industry based upon this discovery has come into being in which ground, ungerminated castor oil seed is mixed with an ordinary fatty oil and a small quantity of acid (preferably acetic). The fatty matter is almost completely hydrolyzed to fatty acid and a very pure glycerol. The reaction takes place rapidly and just a little above room temperature.

Taylor (26) using castor bean lipase and one-fourth more than the molecular quantity of glycerol necessary to produce a triglyceride, was able to synthesize triolein from glycerol and excess fatty acid normal to fat by allowing the enzyme to contact the substrate for six months. He was able to separate the neutral fat produced and identified it.

Twitchell (27) found what he called a "synthetic lipase" (naphthalenestearosulfonic acid) which would cause fatty acid and glycerol to condense in presence of water.

The experimental fact that lipase did not produce complete hydrolysis led Dunlap and Gilbert (28) to successful attempts to synthesize fats through the action of oil-free castor lipase on a glycerol-fatty acid substrate. They were able to show a disappearance in eleven days of more than twenty-six per cent of the total oleic present. They also used glycerol in excess of the amount theoretically necessary to esterify the oleic acid. Synthesis was measured by decrease in acid value.

Welter (29) also synthesized fats from fatty acids and glycerol with castor lipase, and Welter and Ivanov (30) have shown experimentally that the synthetic agent was a lipase.

Armstrong and Gosney (31) in enzyme studies on "The Correlation of Synthetic and Hydrolytic Activity" of castor lipase found that using oleic acid and glycerol in absence of all but traces of water, thirty-eight per-cent oleic acid combined as glyceride in 70 hours. They found that glyceride synthesis diminished with increase in water content and increased with an increase in glycerol content.

Solander (32) obtained eleven per-cent synthesis of oleic acid and glycerol in twelve days using castor lipase.

Sym (33) developed a method for synthesizing esters using pancreas powder and a substrate in which the products are removed from the place of formation as soon as they are formed. This is done by using a medium such as carbon tetrachloride or benzene to dissolve the ester as it is formed at the interface of the aqueous substrate. Many esters have been synthesized through the agency of enzymatic catalysis by this technique including esters of cholesterol butyrate, butyl benzoate, etc., and Rona and associates (34) synthesized wax (cetyl palmitate) by this same method.

The studies set forth in this first section of the present paper have been confined to the synthesis of glycerides from glycerol and a mixture of fatty acids as obtained from the hydrolysis of linseed oil. Enzyme preparations from both plant and animal sources as well as Twitchell's "synthetic lipase" were used to furnish the catalyst to effect synthesis. The active catalyst was assumed to be the enzyme lipase in the case of the plant and animal preparations.

Briefly, the method used was as follows: A substrate composed of various proportions of glycerol, linseed oil fatty acids and water was prepared. To this substrate was added an enzyme preparation. This was then thoroughly mixed and an aliquot portion removed to another flask and weighed, dissolved in ninety-five per cent ethyl alcohol and titrated for its acid number with tenth-normal sodium hydroxide. This gave the free fatty acid content of the original substrate. Then the substrate was kept at a constant temperature and constantly stirred. An aliquot portion was removed every twenty-four hours and the acid number was again determined as before. A decrease in the acid number of the substrate, i.e. the decrease of free fatty acid content, was taken as a measure of the extent of glyceride synthesis assuming that the decreased portion of the free fatty acids combined with some of the glycerol forming a glyceride.

One of the first major problems was to find a good source of lipase which would give maximum synthesis. Of the various sources of lipase it seemed probable from the results of other workers that castor beans and pancreatin powder would be most suitable for this. So, having access to more castor beans than to pancreatin at the time, it was decided to employ lipase from castors first.

Yishio Tanaka in 1910 (35) prepared a lipase from castor beans which was said to be very active. As a matter of fact, this preparation had been used industrially for the hydrolysis of oils and is said to have yielded a high percentage of very pure glycerol. Oil digested at thirty to thirty-five degrees

Centigrade with three to four per cent of the dried enzyme powder and six to ten times as much water as powder yielded ninety per cent glyceride hydrolysis within seven to ten hours.

Armstrong and Gosney (36) used a modification of Tanaka's preparation with good results in studies on the synthetic powers of lipase on a substrate containing glycerol and fatty acids derived from the hydrolysis of olive oil.

It was decided therefore that perhaps this preparation should work well for the problem at hand, so some of it was prepared according to the above methods. To prepare this castor powder the oil is first removed either by ether extraction or by pressing and the residue is then digested with dilute acid. It is then washed thoroughly with water to remove all water-soluble matter and the pasty mass is then dried at a temperature of not more than forty degrees Centigrade.

While this oil-free preparation was being made, preliminary work was carried out using castor beans containing their original oil content. This was done in the first place because several workers had previously noted that lipase may lose its activity in aqueous solution in absolute absence of oil and in the second place because of the belief that lipase would effect synthesis in preference to hydrolysis if placed in the proper substrate even in the presence of oil. The glycerol-fatty acid-water substrate in this case was about sixty times the volume of the oil present in the castors used.

Furthermore, in work on "The Synthesis of Fats by the Action of Enzymes" by Dunlap and Gilbert (37) a large excess of glycerol was used in the substrate with good results. So, as a starting point, a glycerol-fatty acid ratio of three to one was

tried first. Reports in the literature seemed to indicate that castor lipase apparently worked only in an acid medium. There was some conflict of opinion on this point however and some workers found that castors treated first with dilute acid contained active lipase even if not used in a substrate of acid pH.

In preliminary experiments using *Ricinus Zargus* castor beans containing their original oil content the results (Tables I and II) indicated:

1. The catalyst effecting synthesis is apparently an enzyme because heat destroys its synthetic action.
2. This enzyme is not found free in the castor beans but exists as a zymogen because untreated castors give negative results whereas acid-treated castors give positive results.
3. Better results are obtained if the temperature is kept constant at about thirty degrees Centigrade.

The enzyme preparation used in these experiments was prepared by grinding the fine, oily meal, obtained by grinding castor beans in a food chopper, with approximately one-tenth normal sulfuric acid in proportion of 50 grams of the meal to 150 cc of the acid. This mixture was then shaken in a flask for about 15 minutes and then allowed to stand about 5 minutes until the hulls settled after which the upper portion was decanted off, filtered, washed with water and the residue air-dried at from 32 to 37 degrees Centigrade overnight.

The substrate ratios as given in the following tables are indicated as G-A-W, denoting the ratio of glycerol-fatty acid-water.

TABLE I

PRELIMINARY EXPERIMENTS WITH RICINUS ZARGUS CASTORS
CONTAINING THEIR ORIGINAL OIL CONTENT

SUBSTRATE RATIO G A W cc cc cc			TEMP. C.	FATTY ACID CONTENT %	FATTY ACID DECREASE	PER CENT DECREASE	ENZYME USED	TIME DAYS	REMARKS
90 30 15 (6- 2-1)			48	21.26	-	-		0	Reduction
			40	19.49	-1.77	8.23	5 grams H ₂ SO ₄ - treated	1	assumed
			31	19.19	-2.07	9.62	castors	2	due to
			35	19.13	-2.13	10.00		3	glyceride synthesis
90 30 15 (6- 2-1)			48	23.52	-	-	5 grams	0	
			40	23.37	-0.15	neg.	H ₂ SO ₄ - treated	1	Enzyme
			31	23.65	-0.13		castors	2	destroyed
			35	23.56	-0.04		heated to 70°C.	3	by heat
90 30 15 (6- 2-1)			48	22.38	-			0	No
			40	22.36	-0.02	neg.	5 grams castors	1	synthetic
			31	22.18	-0.20		no treatment	2	action
			35	-	-			3	(zymogen)

TABLE II

PRELIMINARY EXPERIMENTS WITH RICINUS ZARGUS CASTORS
CONTAINING THEIR ORIGINAL OIL CONTENT

SUBSTRATE RATIO G A W cc cc cc			TEMP. C.	FATTY ACID CONTENT %	FATTY ACID DECREASE	PER CENT DECREASE	ENZYME USED	TIME DAYS	REMARKS
90 30 15 (6- 2-1)			30	21.42	-	-		0	Reduction
			29	18.41	-3.01	14.05	5 grams H ₂ SO ₄ - treated	1	assumed
			28	16.75	-4.67	21.81	castors	2	due to
			29	15.63	-5.79	27.00		3	glyceride synthesis
90 30 15 (6- 2-1)			30	22.10	-		5 grams	0	
			29	22.08	-0.02	neg.	H ₂ SO ₄ - treated	1	Enzyme
			28	21.94	-0.16		castors heated	2	destroyed
			29	21.46	-0.54		to 70°C.	3	by heat
90 30 15 (6- 2-1)			30	20.89	-			0	No
			29	20.68	-0.21	neg.	5 grams castors	1	synthetic
			28	21.18	0.29		no treatment	2	action
			29	20.89	0.00			3	(zymogen)

In other preliminary experiments (Table III) more than twice the amount of free fatty acid disappeared from the substrate when acid-treated, oil-free *Ricinus Zargus* castor powder was used with the same G-A-W ratio. It was further reaffirmed in these experiments that the catalyst effecting glyceride synthesis is apparently an enzyme since no changes of fatty acid content of the substrate (within range of the experimental error involved) occurred when the enzyme preparation was heated to 70°C. before use. Results again revealed that dilute acid treatment is necessary to free the active principle involved.

The castor enzyme preparations used in these experiments were prepared as follows: Oil-free castor bean powder was first prepared by exhausted extraction of the ground, *Ricinus Zargus* castor beans with ethyl ether by means of the Soxhlet technique. Five grams of this oil-free castor bean powder were ground in a mortar with 25cc of approximately tenth-normal sulfuric acid. This was then shaken 15 minutes, incubated one hour at 37° C. and filtered. The residue was then washed two or three times with water and allowed to drain to a semi-wet condition. The moist residue was employed. This consisted in each case of about thirteen grams of moist residue containing originally five grams of the oil-free castor meal.

It was also found that Merck's Pancreatin powder (untreated) gave results of about the same magnitude as the acid-treated, oil-free castors under similar conditions. Results showed that Pancreatin contains an enzyme capable of catalyzing glyceride synthesis and that this enzyme exists in the free state in this preparation.

EXPERIMENTS WITH OIL-FREE RICINUS ZARGUS CASTORS.

AND MERCK PANCREATIN POWDER

SUBSTRATE RATIO G A W cc cc cc			TEMP. C.	FATTY ACID CONTENT %	FATTY ACID DECREASE	PER CENT DECREASE	ENZYME USED	TIME DAYS	REMARKS
90 30 15 (6- 2-1)			26	16.50	-	-		0	Reduction assumed
			25	8.79	7.71	47.1	5 grams H ₂ SO ₄ - treated	1	due to
			25	8.72	7.78	47.2	castors	2	glyceride
			26	8.73	7.77	47.2		3	synthesis
90 30 15 (6- 2-1)			26	20.72			5 grams H ₂ SO ₄ - treated	0	Enzyme destroyed by heat
			25	20.61		neg.	castors	1	
			25	20.85			heated to 70°C.	2	
			26	20.90				3	
90 30 15 (6- 2-1)			26	23.19			5 grams castors	0	No synthetic action (zymogen)
			25	22.97		neg.	not treated	1	
			25	23.09			with H ₂ SO ₄	2	
			26	23.25				3	
90 30 15 (6- 2-1)			26	22.48	-			0	Reduction assumed due to glyceride synthesis.
			25	18.46	-4.02	17.9	5 grams Pancreatin powder	1	
			25	14.89	-7.57	33.8	(merck) untreated	2	
			26	11.05	-11.43	50.8		3	
90 30 15 (6- 2-1)			26	21.87	-		5 grams Pancreatin powder	0	Enzyme destroyed by heat
			25	21.66	-0.21	neg.	(Merck) untreated	1	
			25	21.18	-0.69		Heated to 70° C.	2	
			26	20.79	-1.08			3	

Under experimental conditions similar to those just described, soy beans, both acid-treated and untreated, gave negative results. Peanuts and also oil-free linseed meal gave unsatisfactory results as did unripened castor beans. Unripened castor beans were used in which the oil was just beginning to form with the idea in mind that perhaps they would contain more lipase or perhaps some catalyst especially suitable for oil synthesis.

Having obtained positive results with the 6-2-1 G-A-W ratio using Merck's Pancreatin powder and having found, in further preliminary experiments not presented here, that a reversal of the G-A ratio yielded higher results, it was decided to find out just how much of this Pancreatin powder was necessary to obtain maximum synthesis using the 2-6-1 G-A-W ratio. Results (Table IV) indicated that five grams of this powder was optimum quantity for maximum results.

Having finally obtained positive results of over fifty percent reduction in each case with either a G-A ratio of 2-6 or 6-2, attention was now turned to the effect of variation of the water content of the substrate.

It was decided to compare some of these substrate results with results obtained by Armstrong and Gosney (38) who made extensive studies on the effect of water on both hydrolysis and synthesis of glycerides. They found that water had a retarding effect upon both conditions of equilibrium but that apparently at least a film of water is necessary at the interface of the system where interaction takes place and that "if this be removed, action comes to an end."

TABLE IV

EXPERIMENTS USING MERCK PANCREATIN POWDER

SUBSTRATE G-A-W	RATIO G A W cc cc cc	TEMP. C.	FATTY ACID CONTENT	FATTY ACID DECREASE	PER CENT DECREASE	ENZYME USED	TIME DAYS
2-6-1	30 90 15	29	22.29				0
		30	22.66				1
		30	22.41		neg.	0.1g.	2
		29	22.69				3
2-6-1	30 90 15	29	23.00				0
		30	23.24				1
		30	22.54		neg.	0.5g.	2
		29	22.36				3
2-6-1	30 90 15	29	22.25	-	-		0
		30	21.33	0.92	4.08		1
		30	20.51	1.74	7.82	1.0g.	2
		29	19.95	2.30	10.34		3
2-6-1	30 90 15	29	21.49	-	-		0
		30	14.93	6.56	30.50		1
		30	10.71	10.78	50.20	5.0g.	2
		29	9.18	12.31	57.30		3
2-6-1	30 90 15	29	20.31	-	-		0
		30	10.94	9.37	46.20		1
		30	8.99	11.32	55.70	10.0g.	2
		29	8.72	11.59	57.10		3
2-6-1	30 90 15	29	22.71				0
		30	22.71			none	1
		30	22.58		neg.	(blank)	2
		29	23.09				3

Using the same method as they and an enzyme prepared in the same way as outlined by Armstrong and Gosney (38), results were obtained using the fatty acids from linseed oil which were very similar to the results of their work in which fatty acids from olive oil were employed. This comparison of results is shown in Table V and in Graph I.

The enzyme preparation used in these experiments was prepared as follows: Fifty grams of mature, fresh castor beans (decorticated) were ground in a mortar with a little petroleum ether. This ground residue was then extracted eight hours with petroleum ether using the Soxhlet technique. It was then allowed to stand in petroleum ether overnight and the residue was then further extracted another eight hours as just described using ethyl ether. Having stood over night in ethyl ether, the residue was then air-dried, ground again in a mortar with ethyl ether and centrifuged. This residue was air-dried and ground to a powder in a mortar. The yield of 14.5 grams of dry powder thus obtained was treated with 120 ml of approximately tenth-normal acetic acid for twenty minutes at 30° C. and filtered. The residue was washed with water and then dried at 37° C. 24 hours after which it was again powdered in a mortar. The final yield was seven grams of powder.

Results were negative using fatty acids from linseed oil and sulfuric acid-treated castor beans containing their original oil content with each of the following: pentaerithritol, maltose, α -methyl-d-glucoside, dextrose, levulose and galactose.

TABLE V

RESULTS USING ARMSTRONG AND GOSNEY MODIFICATION
 OF TANAKA'S ENZYME PREPARATION
 WITH GLYCEROL AND LINSEED OIL FATTY ACIDS

SUBSTRATE RATIO G A W cc cc cc			TEMP. C.	FATTY ACID CONTENT %	FATTY ACID DECREASE	PER CENT DECREASE	ENZYME USED	TIME HOURS
3 30 0 (1-10-0)			29	80.8	-	-		0
			29	77.7	3.1	3.8		1
			29	71.4	9.4	11.6		6
			29	64.6	16.2	20.0	3 grams	24
			29	52.6	28.2	34.9	castor	48
			29	51.5	29.3	36.2	powder	72
3 30 0 (1-10-0)			29	80.6	-	-	Blank	0
			29	82.3	1.7	-	using	1
			29	81.0	0.4	-	2 grams	6
			29	76.0	4.6	5.7	powder	24
			29	77.0	3.6	4.5	heated	48
			29	77.8	2.8	3.4	to 93°C.	72

GRAPH I

COMPARISON OF RESULTS REPORTED BY ARMSTRONG AND GOSNEY WITH
RESULTS OBTAINED IN PRESENT WORK

Whether an enzyme reaction is one say of hydrolysis or one of synthesis depends among other things upon the ratio of the components of the substrate. For example, when dealing with hydrolytic enzymes we should perhaps be able theoretically to increase synthesis by decreasing the water content of the substrate in the presence of stoichiometric proportions of the other components.

It is obvious here that a catalyst may accelerate the reaction in either direction according to the conditions of the substrate. The concentrations of the substrate components are the main, deciding factors concerning whether hydrolysis or synthesis occurs. Also, according to the Law of Mass Action, which states that the rate of reaction between any two substances in a mixture is proportional to the product of the active masses of these substances,

$$\frac{(A) \times (B)}{(AB) \times (H_2O)} = K \quad \begin{array}{l} \text{If } (H_2O) \text{ increases, then } (A) \times (B) \text{ increases.} \\ \text{If } (H_2O) \text{ decreases, then } (AB) \text{ increases.} \end{array}$$

From a theoretical standpoint therefore, considering these and other mechanisms of reversible enzyme reactions, one can surmise that synthesis through the catalytic agency of an enzyme may be a possibility if the concentrations of the substrate components are controlled.

If one considers an enzyme reaction according to the old, key and lock conception of Fisher, then he might expect that possibly, under proper conditions, an enzyme such as lipase which

will unlock a glyceride such as linseed oil, may also be made to re-lock the components. Much work has been reported in the literature, especially in recent years, to substantiate the fact that enzymes do effect synthesis under proper substrate conditions.

In further extensive work therefore, an attempt was made to find out just what conditions are necessary in order to cause lipase to synthesize glycerides of linseed oil fatty acids.

As we have seen from theoretical considerations, the absence of water should cause synthesis to proceed in a reversible reaction of the type mentioned. This particular point, i.e. the effect of the presence or absence of water in a substrate on enzymatic synthesis, is one of the major discrepancies among conflicting reports of workers in this particular field.

In so many cases in enzyme work of this kind it seems that the facts of experimentation throw out many nice, theoretical explanations concerning the possible mechanisms involved in the process. This was found to be the case in the present work, since the use of stoichiometric quantities of substrate components did not always give maximum results. Apparently one reason why the Law of Mass Action is not always applicable in enzyme reactions is the fact that apparently so many of these reactions occur at the interface of colloid surfaces where the actual concentrations are not easily controlled.

Table VI shows the effect of various amounts of water on a G-A ratio of approximately stoichiometric proportions for combination of the fatty acids and glycerine as triglyceride. Note however that the results here are not as high as those obtained with the same type of enzyme preparation and the 6-2-1

ratio as previously noted in Table III where the results were about 47% decrease of free fatty acids.

It was felt however that some ratio of substrate components would yield maximum results of synthesis and therefore work was planned in a purely exploratory fashion to find the best conditions for obtaining glycerol synthesis of the particular problem at hand regardless of theory.

Some thirty-six experiments were run including some two hundred separate substrates in which various substrate ratios were used with various enzyme preparations.

Table VII contains a composite of positive results from these experiments listed in declining order. These positive results, along with indifferent results (in which a decrease of fatty acid was followed by either less decrease or an increase) and with negative results, were compiled together for each different enzyme used so that the G-A-W ratio giving maximum synthesis could be ascertained for each particular enzyme preparation. An example of this is shown in Table VIII. Conclusions concerning the other enzyme preparations were arrived at in the same way.

After correlation of all data obtained in this present work it was found that each type of enzyme preparation gives a maximum yield with a definite G-A-W ratio of its own.

It is evident (Table VII) that of the various G-A-W ratios tried with each particular enzyme, the following general conclusions may be drawn:

1. The 1-10-15 G-A-W ratio is apparently best suited for the oil-free, acid-treated castors. (Maximum yield 57.7% fatty acid reduction.)
2. The 3-1-1.6 G-A-W ratio apparently gives best results with the dilute sulfuric acid-treated Ricinus zargus

castors containing their original oil content. (Maximum yield obtained: 51%)

3. Merck's Pancreatin powder apparently works best with a 2-6-1 G-A-W ratio using five to ten grams Pancreatin powder. (Maximum yield obtained: 57%)
4. The 3-1-1.6 G-A-W ratio works favorably with Twitchell's Naphthalenestearosulfonic acid ("synthetic lipase") (Maximum reduction: 32%)
5. A 37% yield is the best obtainable with mixed variety castors both acetic and sulfuric acid-treated.

In several substrates which were submitted for confirmation of the esterification produced and for the determination of the type of glyceride formed, results of the DeVoe and Reynolds Research Laboratory indicated that in those substrates used for analysis, esterification had taken place and that a monoglyceride had apparently been formed.

Thus, the results of this present work indicate that esterification of glycerides by enzymatic catalysis is possible in vitro if the proper ratio of substrate components is used. No set rules however may necessarily be applied in order to calculate the best ratio to use for maximum results by each type of enzyme preparation.

In summary therefore, it has been found in this work:

1. Using linseed oil fatty acids and glycerol, glycerides have apparently been synthesized through the enzymatic catalysis of lipase from Pancreatin powder (Merck) and of lipase liberated by dilute acid treatment of castor beans.

2. Naphthalenestearosulfonic acid will also catalyze this synthesis.
3. Each different catalyst requires a definite G-A-W ratio in order for synthesis to occur.
4. water up to a certain amount may be present in these synthetic reactions.
5. Castor beans may be used with or without their original oil content if treated first with dilute acid to liberate the catalyst.

TABLE VI

EFFECT OF VARIOUS AMOUNTS OF WATER ON
A SUBSTRATE OF GLYCEROL-FATTY ACID (LINSEED OIL) RATIO OF 1-10

SUBSTRATE RATIO G A W cc cc cc	TEMP. C.	FATTY ACID CONTENT %	FATTY ACID DECREASE	PER CENT DECREASE	TIME DAYS	ENZYME USED
12-120-0	30	83.0	-	-	0	5 grams
(1-10 -0)	30	71.5	11.5	13.8	1	oil-free
	30	72.5	10.5	12.6	2	acetic acid-
	30	85.0	-	-	3	treated
						castor
						powder
10-100-10	30	68.0	-	-	0	5 grams
(1-10 -1)	30	65.5	2.5	3.6	1	oil-free
	30	68.0	0.0	0.0	2	acetic acid-
	30	69.5	-	-	3	treated
						castor
						powder
8-80-40	30	54.5	-	-	0	5 grams
(1-10-5)	30	52.5	2.0	3.6	1	oil-free
	30	53.5	1.0	1.8	2	acetic acid-
	30	54.5	0.0	0.0	3	treated
						castor
						powder
5-50-75	30	32.3	-	-	0	5 grams
(1-10-15)	30	31.2	1.1	3.4	1	oil-free
	30	24.6	7.7	23.8	2	acetic acid-
	30	20.6	11.7	36.2	3	treated
						castor
						powder
3.6-36-90	30	21.2	-	-	0	5 grams
(1-10-25)	30	19.2	2.0	9.4	1	oil-free
	30	20.4	0.8	3.7	2	acetic acid-
	30	20.4	0.8	3.7	3	treated
						castor
						powder
2-20-100	30	13.0	-	-	0	5 grams
(1-10-50)	30	14.5	-	-	1	oil-free
	30	13.8	-	-	2	acetic acid-
						treated
						castor pwd.

TABLE VII
POSITIVE RESULTS

SUBSTRATE RATIO G* A- W	TIME (DAYS)			TEMP. C°	ENZYME USED
	1.	2.	3.		
3- 1-1.6	25.5	55.6	80.5	28	5g. Decorticated R.Z.castors oil & H ₂ SO ₄
2- 6-1	46.2	55.7	57.1	30	10g. Merck Pancreatin powder
2- 6-1	30.5	50.2	57.3	30	5g. Merck Pancreatin powder
1-10-15	10.4	33.9	57.7	30	1g. Oil-free, HAC castor powder
3- 1-1.6	22.6	46.3	51.2	30	5g. R.Z.castors, oil & H ₂ SO ₄
6- 2-1	17.9	33.8	50.8	25/26	5g. Merck Pancreatin powder
1-10-15	48.3	48.3	48.9	30	1g. Oil-free, HAC castor powder
6- 2-1	18.0	38.3	47.3	30	5g. Merck Pancreatin powder
6- 2-1	47.1	47.2	47.2	25	13g. R.Z. oil-free, H ₂ SO ₄ castor powder
1- 3-2	6.3	9.1	44.9	30	5g. R.Z. castors, oil, H ₂ SO ₄
3- 1-1.6	39.1	39.7	41.0	30	10g. Baker's R.Z.castors, oil, H ₂ SO ₄
6- 2-1	27.3	36.2	40.3	28/31	5g. Decorticated R.Z.castors, oil, H ₂ SO ₄
1- 9-15	-	1.6	40.0	39	5g. Merck Pancreatin powder
3- 2-1	37.4	37.7	37.6	30	10g. Mixed castors, oil, HAC
1-10-15	3.4	23.8	36.2	30	5g. Oil-free, HAC castor powder
1-10-0	20.0	34.9	36.2	29	3g. T.A.&G. oil-free, HAC mixed castor pwd.
3- 1-1.6	24.2	34.4	35.2	30	5g. Gould Pancreatin powder
2- 6-1	28.4	35.2	-	30	5g. Baker's R.Z.castors, oil, H ₂ SO ₄
3- 1-1.6	23.7	39.3	34.7	30	5g. Mixed castors, oil, H ₂ SO ₄
2- 3-1	20.4	30.2	33.3	30	2g. Pfanstiehl Steapsin powder (lipase)
3- 1-1.6	13.9	25.4	32.1	30	5g. Naphthalenestearosulfonic acid.
2- 6-1	18.8	25.0	31.5	30	10g. Gould Pancreatin powder
3- 1-1.6	14.2	23.4	30.1	30	5g. Naphthalenestearosulfonic acid.
6- 2-1	10.8	22.9	30.1	31/33	5g. Merck Pancreatin powder
2- 3-1	24.3	28.3	29.7	30	10g. Mixed castors, oil, H ₂ SO ₄
3- 1-2	23.2	22.4	29.6	30	5g. Oil-free, HAC castor powder
1-10-15	-	6.1	27.1	30	5g. Oil-free, HAC castor powder
6- 2-1	14.1	21.8	27.0	29	5g. R.Z. castors, oil, H ₂ SO ₄
2-6- 1	11.1	19.0	24.7	30	5g. Merck Pancreatin powder
3- 1-1.6	18.0	22.1	24.5	30	10g. Gould Pancreatin powder
1-10-50	0.0	20.1	24.3	30	5g. Oil-free, HAC castor powder
3-1 -1.6	9.6	15.5	24.4	30	5g. Merck Pancreatin powder
1-10-0	5.8	11.4	24.0	30	5g. Mixed castors, oil, HAC
1-10-15	-	16.5	23.6	40	5g. Merck Pancreatin powder
2- 6-1	11.2	18.3	21.0	30	10g. Gould Pancreatin powder
2- 6-1	6.8	14.0	21.2	30	5g. Naphthalenestearosulfonic acid
2- 3-1	10.9	18.1	20.5	32	5g. R.Z. castors, oil, H ₂ SO ₄
3- 2-1	9.3	15.8	20.1	30	5g. Merck Pancreatin powder
2- 6-1	5.2	11.7	17.6	30	5g. Naphthalenestearosulfonic acid
2- 6-1	15.5	16.0	16.4	30	5g. Oil-free, HAC castor powder
2- 6-1	7.4	12.2	15.9	30	5g. Gould Pancreatin powder
2- 3-1	6.7	10.6	14.8	30	5g. Merck Pancreatin powder
1-9 -25	4.2	6.0	14.2	39	5g. Merck Pancreatin powder
6- 2-1	8.6	11.6	13.6	30	5g. Mixed castors, oil, H ₂ SO ₄
2- 6-1	11.5	11.5	13.1	31/32	12g. Mixed castors, oil, H ₂ SO ₄
2- 6-1	13.0	13.0	13.0	30	10g. Mixed castors, oil, H ₂ SO ₄
3- 1-1.6	4.9	9.4	12.5	30	10g. Naphthalenestearosulfonic acid
2- 3-1	4.3	7.7	11.3	30	5g. Oil-free, HAC castor powder
6- 2-1	9.1	10.1	11.2	32	5g. R.Z. castors, oil, H ₂ SO ₄
2- 6-1	4.1	7.8	10.3	30	1g. Merck Pancreatin powder

ENZYMATIC SYNTHESIS OF SUGAR ESTERS
FROM DEXTROSE AND LINSEED OIL FATTY ACIDS

Having obtained more or less successful results in bringing about synthesis of glycerides through enzymatic catalysis, attention was now turned to the problem of getting linseed oil fatty acids to combine with derivatives of polyhydric alcohols by similar means.

It was felt at the beginning that definite conditions would have to be worked out for this particular reaction through the use of the exploratory method, as was found necessary in the synthesis of glycerides, and that possibly an enzyme other than lipase may have to be employed.

Nothing was found in the literature concerning any work like that contemplated. It was hoped however that information obtained from the glyceride studies would be of value for finding the proper ratio of substrate components in this present work. There was some doubt nevertheless that previous work would be of any great value in this problem since actually this is an altogether different reaction. But insight gleaned from the work on glycerides did point more or less to the major factors which would most probably influence this reaction for obtaining maximum synthesis or which might contribute to its inhibition.

In the present work the dextrose presented several problems not encountered in the glyceride work. In the first place, the dextrose had to be dissolved and there arose the possibility that certain forms of it may react whereas others may not and thus the possible effect of mutarotation and the time factor between solution of the dextrose and its use became involved. The possibility of fermentation of the dextrose presented another problem and of course the variation of dextrose concentration with respect to the other substrate components had to be considered. It was also

much more difficult to obtain and maintain an emulsion, even temporarily, in the present case compared to the experiments in which glycerol helped greatly to stabilize the substrate in this respect.

Therefore, the present work posed many more problems and the mechanism of this particular reaction became more involved, more difficult to work with and less tangible of accurate interpretation than the previous problem of glyceride synthesis.

Several different factors which might possibly influence the reaction were studied simultaneously in many individual experiments. For example, the effect of water content, concentration of dextrose and fatty acids, temperature, the order of adding components together in preparing the substrate, age of the dextrose solution, source and use of the enzyme, effect of stirring etc. were studied.

As a starting point, G-A-W- (glycerol-fatty acids-water) ratios which had been used and which were found previously to give best results in the glyceride synthesis were used as standards with which to compare possible reactions of D-A-W (dextrose-fatty acid-water) ratios of similar proportions and, using these, the effect of water content and variation of dextrose concentration was studied. Knowing the maximum yield of glyceride obtainable with Pancreatin powder, the blanks or controls of G-A-W substrates were run as a check to see if the enzyme preparation used each time was up to par.

The following pages describe typical experiments of comparative runs in these studies.

Comparative runs were made using five grams of Merck's Pancreatin powder and a 1-9 G-A ratio with variation of water

content and similar substrates containing dextrose in the place of the glycerol. The "D" in this series was a 90% aqueous solution of Pfanstiehl dextrose (dissolved with the aid of heat). Substrates were made so that the total volume was approximately the same in all cases even though substrate ratios were different. Dextrose was in excess of stoichiometric quantity in numbers 7, 8 and 9, but stoichiometric proportions of dextrose and fatty acids existed in number 6.

Substrate			Results after 3 days at 39° C.			
#	G-	A- W	% FA	no	% FA	Enzyme
			decrease	indifferent change	increase	
1	1	9 0			17.8	Five grams
2	1	9 5		X		
3	1	9 10	3.1-----X			Merck's
4	1	9 15	40.0			
5	1	9 25	14.2			Pancreatin
						powder
6	0	9 5			5.5	
7	0	9 10		X		used
8	0	9 15		X		
9	0	9 25		X		in each

Upon addition of 25 ml more of water to each of numbers 6, 7, 8 and 9 there appeared no significant decrease after four more days of incubation (3.1% decrease only, in #8). Upon addition of still more water and five grams more of Pancreatin powder to each of numbers 1,6,7,8 and 9, no changes occurred after three days further incubation at 37-38° C. except an apparent 22.8% decrease after two days in substrate number 1.

Since according to previous experiments with glyceride synthesis the 2-6-1 G-A-W ratio is apparently optimum for Pancreatin powder (57.3% reduction in fatty acids resulted in three days) this ratio was tried. Experiments employing both 90% and 85% dextrose solution instead of the glycerol resulted in either indifferent or negative action. The order in which the

substrate components were added together was also studied but no definitely positive results occurred in any case. This was equally as true when castors were used with optimum ratios. One exception occurred however in which 9 grams of fatty acids and 25 ml of water containing 4 grams dextrose were incubated with R.Z., H_2SO_4 -treated castors at $30^{\circ}C$. giving decreases of 3.3%, 7.9% and 18.3% in 24, 48 and 72 hours respectively.

It is obvious from the few experiments shown above that such comparative runs gave quite erratic results insofar as the dextrose substrates were concerned.

From these and many other similar experiments in comparative studies concerning the substitution of dextrose in place of glycerol, the following conclusions were drawn.

Using substrate ratios and enzyme preparations which were found in previous work to give optimum results with glycerol, results were mostly negative when dextrose was substituted for glycerol in the substrate. Furthermore, many such results were indifferent and many others even increased in acid number. The few results showing any appreciable reduction in acid number were not capable of being reproduced at this time.

In order to get satisfactory, temporary emulsions for obtaining homogeneous and representative aliquots for determining the acid number every 24 hours, very concentrated solutions of dextrose were used in most cases. This may possibly have destroyed or denatured the enzyme by dehydration. In most cases when dilute solutions of dextrose were used, homogeneous aliquots could not be obtained and results were therefore erratic.

In view of the above results and conclusions, it was decided that the general method which was successful with glycerol was apparently not ideally applicable when dextrose was used.

It was felt that perhaps in order for optimum sugar ester synthesis to occur, the substrate components, i.e. dextrose and fatty acids, should be in closer contact with each other.

In looking back over the literature of fat and ester synthesis by enzymes it was found that in several cases organic solvents were used as mutual solvents for the substrate components apparently with good results. (39-48)

One noteworthy example of these organic solvent substrates is the use of acetone. It is claimed that lipase for example will work in an acetone medium if a certain amount of water is present.

In accordance with this idea, proportions were worked out for acetone and water which would hold in solution both fatty acids and dextrose of approximate molarity. This therefore offered one possibility for the enzymatic synthesis of dextrose with linseed oil fatty acids if the proper enzyme could be found to catalyze the reaction.

Another example of the use of an organic solvent in this work is that of Sym. Many esters are reported having been synthesized by this method. Rona and co-workers have even claimed to have synthesized wax (cetyl palmitate) by using this technique. In the case of the problem at hand, using the method of Sym, the fatty acids could be in solution in benzene or a similar solvent and the dextrose could be in solution along with the enzyme in the aqueous phase. Sym claims that synthesis takes place in the water of imbibition of the enzyme which concentrates itself in the aqueous phase near the phase interface. The product of the reaction is

removed as soon as it forms by reason of its solubility in the organic solvent thus removing it from the site of synthesis. The acid number of the organic solvent is measured to follow the extent of synthesis. The organic solvent thus acts as a reservoir for the fatty acids and also removes the reaction product from the place of formation thus causing the reaction to proceed forward. In the present case, any concentration of dextrose could be used which would not interfere with the action of the enzyme.

It was felt however that perhaps an acetone solution containing both the fatty acids and the alcohol (glycerol) or the polyhydric alcohol derivative, together with Pancreatin, might be an ideal way to bring the active substrate components into more intimate contact in a homogeneous manner, therefore this was tried first.

Proportions were worked out to ascertain first the amount of water that could be added to a solution of fatty acids in acetone and still retain a clear solution. Then the amount of either glycerol or dextrose that could be dissolved in this dilute acetone solution of fatty acids was determined for various concentrations of fatty acids.

These results were then made into graphs and from the curves obtained, proportions were found in which a stable, clear, apparently true solution of dilute acetone resulted containing glycerol and fatty acids or dextrose and fatty acids in concentrations of similar molarity.

In one experiment for example, the substrate contained 0.3M fatty acids and 0.2M dextrose in acetone containing the maximum amount of water. Such substrates were run containing various amounts of Pancreatin powder(0.1g., 0.2g., 0.5g., 2.0g., 3.0g., 5.0g.).

Results of these however were negative and increases in acid number occurred in some cases.

In another experiment, the substrate contained a solution of 0.35 M fatty acids and 0.35 M glycerol in acetone containing its maximum content of water to maintain solution. Several such substrates were run at the same time each containing a different amount of Pancreatin powder (0.1g., 0.2g., 0.5g., 1.0g., 2.0g., 3.0g., 5.0g., 10.0g.). All of these were negative except the one containing 10.0g. Pancreatin. In this particular case, a reduction of 17.6% of free fatty acids resulted in 24 hours and in 48 hours a free fatty acid reduction of 22.2% occurred. The substrate in this case was prepared as follows. To 8.1 ml of a dilute glycerol solution (8 g. glycerol dissolved in 75 ml of water resulting in a total volume of 81.0 ml) 10 g. of Pancreatin powder were added and allowed to stand overnight at 37° C. To this was added 25 ml of acetone solution of fatty acids (24.5 g. linseed oil fatty acids diluted up to 250 ml with acetone) The whole was then mixed until a homogeneous solution resulted. This was then stirred in a water bath kept at a constant temperature of 37° C. The final substrate contained 25 ml fatty acids in acetone (0.35 M), 7.5 ml water and 0.8 ml glycerol (0.35 M). It is conceivable that this substrate could be improved upon by employing a glycerol extract of Pancreatin instead of using Pancreatin powder which gets quite voluminous when large quantities are employed.

Attempts were now made to utilize the method of Sym in the present problem. Preliminary experiments were run according to the technique of Sym (39-44) using Pancreatin powder, castor bean lipase and various alcohols and acids such as butyl alcohol,

glycerol, oleic acid and benzoic acid in benzene-water substrates. Sodium glycocholate was also used in addition in a few instances. On the whole, only a few slightly positive results were obtained.

In a similar setup using dextrose, linseed oil fatty acids and Pancreatin powder in benzene-water substrate, a slight increase in acid number was noted.

Using glycerol and linseed oil fatty acids, some positive results were obtained employing the method of Sym as is shown in the following examples.

In one case, 25 grams of linseed fatty acids were diluted to 75 ml with benzene and to this was added 15 grams of a glycerol extract of Pancreatin containing 3 ml of 30% sodium glycocholate and 3 ml of 10% sodium acetate. This was kept at 30° C. not stirred. A free fatty acid decrease of 1.7% resulted in one day, 3.8% in two days and 3.8% in three days.

In another case, a setup containing 14 grams linseed fatty acids diluted to 50 ml with benzene, 30 ml 10% sodium acetate, 2 grams Pancreatin powder, 4.6 grams glycerol and 2 ml of 30% sodium glycocholate kept at a constant temperature of 37° C. and not stirred, resulted in a free fatty acid reduction of 6.0%, 7.3% and 12.6% in one, two and three days respectively.

Under similar conditions a 15 ml glycerol extract of Pancreatin, 3 ml of sodium glycocholate and 30 ml oleic acid kept at 37° C. not stirred yielded reduction of 4.4%, 7.6% and 11.8% after one, two and three days incubation.

However, in further similar experiments using the Sym technique, most of the results were practically negative or indifferent when glycerol and linseed oil fatty acids were used

in benzene-water substrates with Pancreatin. Optimum as well as stoichiometric ratios were used in these cases. The only positive result of interest here was one which contained 112.5 ml of water, 4.0 grams dextrose, 10 grams Pancreatin powder, 40.5 ml linseed oil fatty acids and 100 ml benzene. This resulted in a fatty acid decrease in one day of 1.5% of the original fatty acids present and 17.1% after two days.

It is also of interest to note that in another set of substrates which contained 25 grams hog intestine ground with sand in a mortar, 50 ml glycerol, 15 ml water and 90 ml linseed oil fatty acids, results were indifferent in the presence of 5 ml of ox bile but were positive in absence of bile to the extent of 5.3% fatty acid decrease in one day and 17.3% after two days.

It was decided at this time to go back and try to improve upon the original method using an aqueous solution of dextrose together with linseed oil fatty acids and to employ the idea of sym to this by not stirring the two phases except when aliquots were taken for determination of the acid number.

Results of these and previous experiments revealed that apparently optimum conditions would exist if the substrate contained 4 grams dextrose dissolved in 112.5 ml water with 10 grams Pancreatin powder and 40.5 ml linseed oil fatty acids added and the whole kept at a constant temperature of 30° C and not stirred. Similar specimens prepared at the same time but kept at constant temperature of 34.5° C. and others which were stirred all gave negative results. Other similar experiments in which 90% dextrose solution was used yielded negative results.

Results of the optimum conditions in these experiments were

as follows:

days	% free acids reduced	acid number
0	--	23.9
1	6.7	22.3
2	15.5	20.2
3	17.2	17.8

Results of further experiments of the kind just described yielded similar conclusions. More specifically, the best results were given by the following conditions. To 112.5 ml water dissolve 4 grams dextrose. Overlay this solution immediately with 10 grams Pancreatin powder and allow to stand until the powder wets and sinks to the bottom of the flask. Shake well until a good emulsion results. Allow to stand two hours. Then add 40.5 ml fatty acids. Mix well and take aliquot immediately for acid number determination. Allow to stand without stirring at a temperature of 30° C. The following results show the more positive yield thus obtained.

days	% decrease free fatty acids	acid number
0	--	24.4
1	3.3	23.6
2	23.3	18.7
3	27.5	17.7

In similar substrates, when H_2SO_4 -treated castors containing their original oil content were used instead of Pancreatin, unstable emulsions resulted and results were indifferent.

An attempt was made to ascertain the effect of temperature on the substrates containing Pancreatin. None of these substrates were stirred. One was kept at room temperature, one at 37° C. and one was refrigerated at 8° C. Each substrate resulted in a milky emulsion. Each contained 112.5 ml linseed oil fatty acids

112.5 ml water, 10 grams Pancreatin powder and 4 grams dextrose added together in the order: water, dextrose, pancreatin, fatty acids.

Percentage decrease of original free fatty acids resulted as follows:

Room temp.	22-26° C.	Refrigerated at 8° C.	Incubated at 37° C.
1 day	11.7	1 day	11.3
2 days	37.4	2 days	12.3
3 days	27.1	3 days	61.0

Results were negative at 23-24° C. using 100 ml water, 5 grams dextrose, 35 grams fatty acids and 10 grams Pancreatin powder.

Experiments in which formalin was used in an attempt to prevent fermentation yielded results which were inconclusive. Apparently anything that might hinder or prevent the fermentation also hindered or prevented the enzyme action.

A very troublesome problem presented itself when, in following the same procedure in which positive results were obtained, the results would be negative. In other words, in most cases, positive results could not always be duplicated. This led to the belief that perhaps some action in the preparation of some of the substrates was overlooked as to its importance in effecting the reaction and was therefore not noted in the directions for preparation. Therefore, in still another attempt to get more information concerning this, a series of experiments was run to ascertain the effect of the concentration of Pancreatin to use, the advisability of hydrating the Pancreatin before use, the effect of various amounts of formalin on the reaction and on fermentation, the effect of temperature and the effect of mutarotation of the dextrose solution as well as the effect of the order of adding the substrate components. It was believed the latter two effects

were most important. The following results represent typical experiments in which the above effects were studied.

Each substrate contained 112.5 ml of water, 4.0 grams of C.P. dextrose, 10 grams Pancreatin powder and 40.5 ml of linseed oil fatty acids. All substrates were kept at room temperature (23°) and none were stirred. In this series, each of the seven substrates was treated in a different way as follows:

1. All components were added together immediately and the Pancreatin was not hydrated before use.
2. The dextrose solution was incubated overnight and the Pancreatin powder and fatty acids were then added.
3. The dextrose and the Pancreatin powder were incubated together overnight and the fatty acids were then added.
4. The dextrose solution was incubated overnight, the Pancreatin powder was hydrated before use and the fatty acids and formalin were finally added.
5. The Pancreatin powder was first hydrated and was then incubated together with the dextrose overnight. Fatty acids and formalin were then added.
6. The Pancreatin powder was first hydrated. The other components (dextrose and fatty acids) were then added and the substrate was kept at room temperature (23° C.)
7. The dextrose solution was incubated overnight at 25° C. (instead of the incubation temperature of 37° C. that was employed in all of the other substrate preparations). Pancreatin powder and fatty acids were then added.

Results of this series were either negative or erratic, e.g. number 7, after a rise, gave 18.5% decrease the third day.

In another series prepared in the same way as that described above but run for a longer time, the results were generally negative or indifferent with increases resulting the first three days and subsequent decreases in acid values in numbers 2,3 and 4 of about 9% the fourth and fifth days. Those substrates showing more positive results were as follows:

#6 (fermented)			#7 (fermented)		
days	% FA	decrease	days	% FA	decrease
1	3.7		1	0.0	
2	(increase)		2	0.0	
3	(increase)		3	0.0	
4	14.2		4	17.6	
5	12.5		5	12.8	
6	6.9		6	(increase)	
7	(increase)		7	--	

In view of the results obtained in experiments such as those described above, it was finally concluded that the approach just elaborated did not yield accurate information concerning the various effects which were found necessary to be studied.

In order to ascertain the possible effect of mutarotation of the dextrose upon the reaction, a series of substrates was set up which yielded the following results:

substrate	days	%	%	days	substrate
1. Dextrose dissolved in water.	1	- 7.2	- 4.6	1	7. Dextrose dissolved in water. Stood 29 hrs. at 25° C. Pancreatin and fatty acids then added.
wetted pancreatin added immediately.	2	-13.5	-20.5	2	
Fatty acids added.	3	+ 3.9	- 0.9	3	
(fermented)	4	+ 1.9	---	4	
	17	-26.7	-30.7	17	
2. Dextrose in water. Stood 2 hrs. at 37° C. Fatty acids added.	1	+ 8.1	+ 4.6	1	8. Dextrose in water. Pancreatin added immediately. Stood 2 hrs. at 29° C. Fatty acids then added.
(fermented)	2	+ 3.0	+ 7.7	2	
	3	-20.0	+ 1.9	3	
	4	+ 4.0	+ 9.1	4	
	17	- 9.0	---	17	
3. Dextrose in water. Stood 4 hrs at 37° C. Pancreatin and fatty acids added. (fer)	1	+ 2.4	- 5.5	1	9. water, glycerol and Pancreatin added together. Stood 15 min. Fatty acids added. GAW ratio 30-90-15.
	2	- 2.9	- 9.4	2	
	3	0.0	-11.9	3	
	4	+ 2.9	-17.0	4	
	17	-33.3	-33.9	17	
4. Dextrose in water. Stood 6 hrs at 37° C. Pancreatin and fatty acids added. (fer)	1	+ 0.4	+ 1.6	1	10. Dextrose in water. Stood 29 hrs. at 37° C. Pancreatin and fatty acids then added.
	2	+ 3.7	+ 1.6	2	
	3	- 6.0	+ 1.4	3	
	4	+ 3.7	- 2.7	4	
	17	-26.0	---	17	
5. Dextrose in water. Stood 8 hrs at 37° C. Pancreatin and fatty acids then added.	1	+ 1.9	+ 4.8	1	11. Dextrose in water. Pancreatin added immediately. This kept 29 hrs at 37° C. Fatty acids then added.
	2	+ 2.4	+ 3.2	2	
	3	- 1.9	+ 5.8	3	
	4	+ 5.2	- 7.9	4	
	17	- 9.7	---	17	
6. Dextrose in water. Stood 21 hrs at 37° C. Pancreatin and fatty acids then added.	1	+ 0.9			
	2	+ 1.9			
	3	+ 6.8			
	4	---			
	17	-39.2			

In order to check the potency of the Pancreatin powder used in the above experiment, substrate number 9 was repeated using fresh Pancreatin powder. Results revealed the following yield:

<u>days</u>	<u>% FA decrease</u>
1	9.8
2	17.9
3	24.7
4	28.3
17	38.2

The freshness of the Pancreatin powder employed in the subsequent series was assured by checking its potency in a similar way each time before it was used.

Since different sources of dextrose were used in the various experiments, the following series was run in order to determine whether or not dextrose of different purity or whether or not dextrose from the different sources might effect the extent of the reaction. Information from previous experiments was used in order to prepare the best possible substrate for this series.

To 112.5 ml of distilled water (ammonia-free, Permutit-treated) 4 grams of dextrose was added. As soon as it was dissolved, 10.0 grams of fresh, Merck's Pancreatin powder was added. This was allowed to stand (Pancreatin floating on the solution) until it was wetted and sank to the bottom of the solution. It was then mixed thoroughly and 40.5 ml of freshly distilled linseed oil fatty acids were added gradually with constant stirring. This mixture was then mixed thoroughly and a 2.0 ml aliquot was removed and accurately weighed, dissolved in 10 ml of 1% alcoholic phenolphthalein and titrated with 0.1 N aqueous NaOH.

The substrate was kept at a constant temperature and was not stirred except just before an aliquot was taken for fatty acid determination after each 24 hours.

Using Pfanstiehl dextrose the results were as follows:

Temp. C. ^o	% fatty acid decrease			Remarks
	24 hrs.	48 hrs.	72 hrs.	
37	5.2	30.0	24.9	No fermentation
8	10.4	28.0	48.4	No fermentation
25	2.9	7.9	0.5	Fermented
25/26	2.4	2.4	2.8	Fermented

Using "C.P." dextrose the results were as follows:

37	4.8	32.4	28.7	Slight fermentation
8	65.0	33.2	24.8	No fermentation
25	2.8	2.3	5.1	Slight fermentation

In a similar substrate using 4 grams Eastman anhydrous dextrose in 112.5 ml of plain distilled water with 10 grams fresh, Merck Pancreatin powder and 40.5 ml of linseed oil fatty acids, each component added immediately one after the other and shaken thoroughly after each addition and this substrate mixture not stirred, the following results were noted:

days	%FA decrease (80C.)	%FA decrease (37°C)	%FA decrease (boiled)
1	- 2.9	+ 5.7	+ 2.0
2	-17.3	+ 2.8	+ 0.8
3	-41.3	+ 3.3	+ 4.8
4	-30.7	- 4.7	+ 3.2

After due consideration of the data obtained in all of the experimental work done on the present problem, it was felt that probably there might be two different reactions progressing at the same time in the same substrate, each one perhaps progressing at a different rate according to the existing conditions.

It was thought that possibly one of these reactions was a fermentation reaction in which organic acids were being formed, as evidenced in some instances by an increase in the acid number

over that originally present in the substrate and also as evidenced in other cases where the acid number increased again after an initial decrease.

The other reaction, progressin^g at the same time in the same substrate, was hoped to be one of synthesis in which the fatty acids of the substrate were combining in some way with the dextrose, thus giving a gradual decrease in acid number.

It was considered likely that in the light of the above possibilities, the apparently slight decreases in acid number in many cases may possibly have been overshadowed by a counter increase in acids from fermentation and that in many instances the actual total extent of synthesis might have been underestimated since the acid number was the only criterion of measurement concerning the extent of synthesis in these experiments.

Further experiments were therefore planned in an attempt to determine whether or not two reactions were operating simultaneously in this system and at the same time to obtain more specific information concerning the effect of pH, temperature etc. insofar as these are concerned in the control of each reaction with the hope that optimum conditions might be worked out whereby a maximum, progressive decrease in acid number would be obtained while at the same time the fermentation reaction would be suppressed and held at a minimum.

Attempts were made at first to determine the acid number of the aqueous phase each 24 hours in order to ascertain the extent of organic acids formed from fermentation. It was found however that most of the substrate when mixed thoroughly gave temporary emulsions which could not always be sharply and uniformly separated into aqueous and non-aqueous phases and therefore consistent

results were not obtainable. By addition of sodium bicarbonate or phosphate buffers it was thought possible that any organic acids formed might be neutralized as they were formed and thus give the synthesis reaction a better chance to proceed.

Results of this particular series of experiments are best shown and interpreted through the use of graphs.

In the first series, the following quantities of components were used: 112.5 ml water, 4 grams dextrose, 10 grams Pancreatin powder, 40.5 ml linseed oil fatty acids and 1 gram sodium bicarbonate.

The acid number was determined every 24 hours in each of the following different substrates. The substrate components in each case were added in the order given .

1. water, dextrose, Pancreatin and fatty acids.
2. water, dextrose, Pancreatin, NaHCO_3 and fatty acids.
3. water and Pancreatin.
4. water, dextrose and Pancreatin.
5. water and dextrose.

A set of all five of these substrates was kept in three separate series of experiments at temperatures of 37°C . (incubated) in one case, 25°C . (room temperature) in another case and at 5°C . (refrigerated) in still another case.

Comparisons from the graphs (A) resulting from these data show that:

1. The acidity increase in the aqueous phase is apparently not due to fermentation of dextrose but rather to the fermentation of the hydrated Pancreatin powder.

2. Lower temperatures apparently favor the reaction of synthesis in the aqueous substrate. Fermentation was apparently diminished or checked under these conditions.

3. Neutralizing the acidity of the Pancreatin before use, with sodium bicarbonate favors reduction of the acid number of the mixture and therefore it is apparently helpful for synthesis.

In another series of experiments, a phosphate buffer of pH 7.5 was used in place of the water. The following quantities of components were used: 112.5 ml phosphate buffer pH 7.5, 4 grams dextrose, 10 grams Pancreatin powder, 4015 ml linseed oil fatty acids and 1 gram NaHCO_3 .

The acid number was determined separately every 24 hours in each of the following substrates. The components were added in the order given.

1. Buffer, dextrose, Pancreatin and fatty acids.
2. Buffer, dextrose, Pancreatin, NaHCO_3 and fatty acids.
3. Buffer and Pancreatin.
4. Buffer, dextrose and Pancreatin.
5. Buffer and dextrose.

A set of each of these was kept in separate series at temperatures of 37°C ., 27°C . and 5°C .

Comparison of the graphs (B) resulting from these data show that:

1. The dextrose does not ferment but the hydrated Pancreatin does ferment either by itself in the buffer or when dextrose is also added. The results of these two cases are practically parallel.

2. Higher temperature is apparently better suited for this buffered substrate.

3. Neutralizing the Pancreatin before use was also helpful in this medium for obtaining maximum acid number reduction.

In a third series of experiments, phosphate buffers of different pH values were employed and the acid number was run in duplicate in each case.

Each substrate of this series contained the following. The components were added in order given: 112.5 ml phosphate buffer, 4 grams dextrose, 10 grams Pancreatin powder and 40.5 ml linseed oil fatty acids. All were kept at 37° C.

Results as given in the graphs (C) revealed that apparently the reaction of synthesis may work best in a slightly alkaline medium. Soaps formed may have had a catalytic effect in these instances.

Although it was realized that a mixture of water and fatty acids alone would yield erratic results as far as acid numbers were concerned, due to the fact that a homogeneous aliquot could not be obtained, nevertheless, this was attempted and results as shown in the graphs (D) indicate that consistent results were impossible to obtain. Similar runs (E) using phosphate buffer of pH 7.5 yielded results which were not quite so erratic.

It is to be concluded then that two systems are progressing simultaneously in such substrates as those described above. This perhaps means then that the results as given previously represent somewhat conservative estimates as to the extent of synthesis.

Concerning synthesis of sugar esters by enzymatic catalysis it may be said in summary that attempts were made to apply techniques found previously to be successful for glyceride synthesis but results were erratic and not dependable. The use of the Sym technique and other methods in which organic solvents-water phases were employed also gave for the most part negative results. Some apparently positive results were obtained by employing the aqueous-fatty acid phase mixture under specially developed, improved conditions. Various factors were studied concerning their effects on the synthesis but no particularly definite conclusions could be

drawn since each factor is apparently interrelated with others and no one factor revealed any specific, favorable action to any great extent.

It was finally revealed that the Pancreatin would ferment and form acids. An attempt was made to block this interference and some positive results resulted.

It is admitted that this phase of the present work is incomplete insofar as an exhaustive study is concerned but time has not permitted further work to be done. Results obtained however do point the way to further approaches from which to launch further research into this most interesting and important field.

LEGEND FOR THE FOLLOWING SERIES OF GRAPHS

In each case the abscissa is a measure of days incubation and the ordinate is a measure of the percentage of free fatty acids in the substrate mixture.

In Graphs A and Graphs B:

Color	Substrate Content
Water, dextrose, Pancreatin and fatty acids.
Water, dextrose, Pancreatin, NaHCO_3 and fatty acids.
Water and Pancreatin.
Water, dextrose and Pancreatin.
Water and dextrose.

In Graphs C:

- Average of duplicate runs of phosphate buffers of the pH indicated. All substrates kept at 37° C.

In Graphs D:

- Mixture of water and fatty acids kept at the temperature indicated.

In Graphs E:

- Mixture of phosphate buffer of pH 7.5 and fatty acids kept at the temperature indicated.

37° C.

25° C.

5° C.

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GRAPHS B

54.

37° C.

25° C.

5° C.

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18

16

14

12

10

8

6

4

2

1

0

0

1

2

3

15

0

1

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4

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2

3

4

GRAPHS C

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GENERAL SUMMARY AND CONCLUSIONS

It has been shown in recent years that reversible enzymatic reactions may be controlled so as to cause synthesis to predominate.

Due to the intangible and pioneering nature of the particular problems undertaken in the present work, the exploratory method of research was necessarily employed.

The various factors effecting the synthetic reactions studied have been dealt with in various ways and certain conclusions have been drawn concerning the importance of each factor in its relationship to the others.

It is felt by the writer that glyceride synthesis has been successfully accomplished to a much greater extent than that anticipated.

The work on sugar ester synthesis however has not been so fruitful, but this was rather expected when the writer was assigned this task. Much information has been obtained however in this phase of the present work and it must be realized that in order to completely conquer this particular problem it would require much more time and more ingenious technique than was at the writer's disposal.

This work as a whole is merely a small contribution to the vast lack of knowledge of such enzyme systems. It may be thought of merely as turning the feet to step in the right direction.

In this study of synthesis by enzymatic catalysis the literature has been reviewed and much invaluable information thus gained has led to insight of greater depth into the fascinating realm of enzymology.

Enzymology as it is reviewed by Sumner and Sommers (39), Falk (40), Waldschmidt-Leitz and Walton (41) and Tauber (42;43)

opens a vast and fertile field for Biochemical research. It no doubt will be the center of biochemical interest in the immediate years to come and it offers in its vastness and in its importance a field so large as to offer great possibilities for research efforts for many years to come.

The interrelationships between hydrolysis and synthesis in reversible enzymatic reactions and the important implications of glycolytic, proteolytic and lipolytic enzyme systems in their relationship to various other important enzyme systems is just now beginning to throw brighter light upon the intricate mechanisms involved in metabolism and catabolism of both normal and abnormal conditions.

The clinical use of such enzymes as acid and alkaline phosphatase, lipase, diastase and thrombin has already proven the value of enzyme studies in the diagnosis of pathological conditions and no doubt many more enzymes or enzyme systems will become of clinical value.

Enzyme studies will throw much light upon the cancer problem as the work of Potter and others have shown and indeed in the final analysis the key to the solution of cancer cause and cure lies in the realm of enzymology.

Enzymatic synthesis is now literally growing by leaps and bounds in many phases of biochemical work. Hardly a month passes without some reputable journal including another phase of this intriguing work. For example, the recent work of Green and Stompf (44), Cori and Cori (45), Hidy and Day (46), Doudoroff (47) Colwick and Southerland (48) and others has given impetus to polysaccharide synthesis. The work of Bergmann and Fraenkel-Conrat (49) and others in protein synthesis also points to a general trend toward the reality and possible uses of enzymatic synthesis.

These findings as well as those in the making must eventually become of great importance to medicine, to agriculture, to industry and perhaps to many other related fields.

we now have a new era being ushered in before us in the field of Biochemistry. It is the enzyme age in which these intangibles are becoming approachable by modern methods and techniques, an age which will necessarily influence the philosophy and approach of Physiology and related fields as well as the field of Biochemistry and an age which will in the final analysis definitely effect our scientific philosophy of life itself and of the Creator of all living things.

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