AN INVESTIGATION ON TRIGLYCERIDE SYNTHESIS IN INTESTINAL MUCOSA OF NORMAL AND BILE DEFICIENT RATS IN VIVO

by

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INTRODUCTION AND REVIEW OF THE LITERATURE

The absorption of lipid has been studied for over one hundred years. As the tools available for biological research have progressed from simple stained preparations for the light microscope to complex tracer studies, so too has the study of lipid absorption evolved and become ever more refined. As refinement occurred, it early became clear that lipid absorption involved many facets, any one of which was difficult to assess because of their complex interrelationships.

Stated briefly, a fragmentation of the subject of lipid absorption into its components would include such facets as: 1. The extent of hydrolysis (both luminal and intracellular). 2. The form in which the lipid is actually absorbed. 3. The means by which the lipid enters the mucosal cell. 4. The biochemical events related to lipid metabolism which occur within the cell. 5. The transport of lipid from the mucosal cells into the circulation, and 6. The role of bile. The proability that many of the above facets vary according to the type of lipid involved limits generalities and has been a factor contributing delay to progress in the field.

Each of the above facets has a history of investigations which have contributed information to its understanding. In some instances the progress has been rapid and the history short. A case in point is the advance towards an understanding of the pathways involved in mucosal glyceride synthesis. In some instances the history has been long and has involved prominent names and long controversies, such as Pflüger, Verzár and Frazer and their respective theories of lipid absorption.

Pflüger (46), working around the turn of this century, came to the conclusion that dietary glycerides were completely hydrolized to glycerol and fatty acids and that the fatty acids were subsequently transformed to soaps with "the help of bile and soda". He believed that only as soap could lipid be absorbed into the mucosal cells of the intestine.

In 1936 Verzár published a monograph entitled "Absorption from the Intestine" (58). This publication marks a period of achievement and accumulated knowledge in the field of absorptive physiology for, as stated in the general preface to the monograph, "In this present series of Monographs on Physiology, it is intended to set out the progress of physiology in those chapters (areas) in which the forward movement is the most pronounced". Surely, Verzár's extensive studies in the field of absorption entitled him to selection as author of the monograph.

Verzár agreed with Pflüger that dietary glyceride must be hydrolized into its constituent fatty acids and glycerol before absorption could occur. He disagreed, however, with Pflüger on the following point: Pflüger had concluded that fatty acids present in the intestinal lumen were converted to soaps; Verzár concluded that the formation of soaps in the lumen was impossible due to characteristic luminal pH values of less than 7 (47). Verzár further concluded (on the evidence of several experiments which involved the diffusion behavior of fatty acid-bile salt combinations) that the fatty acids which were released from dietary glycerides by hydrolysis were absorbed into the mucosal cell as part of a fatty acid-bile salt complex.

These conclusions of Pflüger and Verzár regarding the necessity

of complete hydrolysis prior to absorption were quite generally accepted for thirty years. Some contrary evidence had, however, begun to appear as early as 1927. In 1927, Mellanby wrote, in conclusion to one of his papers (38), "The observation which I wish to emphasize is that a mixture of neutral fat and bile only, to which no lipase has access, is rapidly absorbed from the duodenum and jejunum and less rapidly from the ileum into the lymphatic system of the small intestine".

The growing skepticism in the hypothesis of 'complete hydrolysis' (termed the 'Lipolytic Hypothesis') was exemplified in the following quotation from a review article on lipid absorption which was written by Frazer in 1940 (19): "For several reasons, however, the lipolytic Hypothesis must be regarded as unproven". In 1945, Frazer (22) presented concrete in vitro evidence that the end products of the hydrolysis of olive oil by pancreatic lipase were fatty acids and mono- and diglycerides, rather than fatty acids and glycerol. If a brief aside may be allowed here, it would be well to mention that the final resolution of problems related to the extent of luminal hydrolysis waited until the advent of radioactive tracers as experimental tools. Reiser, et. al., in 1952 (48), studied the absorption of a synthetic triglyceride in which the glycerol was labeled with C^{14} and the fatty acids with conjugated double bonds. Analysis of the lymph lipids led to the following conclusion: Approximately 25 to 46 percent of the ingested triglyceride was completely hydrolized during absorption; the remaining 54 to 75 percent was hydrolized to monoglycerides. This study, as well as others, completed the demise of the Lipolytic Hypothesis.

Let us return to a few last comments upon the work of Frazer. He

stands as a prominent figure in the historical background of lipid absorption. His prominence was associated with his 'Partition Hypothesis', an hypothesis of lipid absorption which was quite popular through the 1940's.

Early 'suggestions' of the Partition Hypothesis appeared in his 1940 review of lipid absorption (19), while a complete discussion of experiments relevant to the hypothesis appeared in a later review in 1946 (21). The essence of the Partition Hypothesis involved the following two postulates: 1. Mono- di- and triglycerides could be absorbed, probably not as molecular dispersions, but in particulate form, and 2. Absorbed fatty acids were removed from the mucosal cell via the portal blood, while the absorbed glycerides were removed from the mucosal cells via the lacteals of the lymphatic system. The latter postulate began to be experimentally disproven as early as 1950 by Bloom, et. al. (8). These authors fed palmitic acid-C^{14} (labeled in the carboxyl position) as the free fatty acid dissolved in corn oil to rats with either thoracic duct or intestinal lymphatic cannulae. They recovered 70 to 90 percent of the absorbed palmitic acid-C^{14} from the intestinal lymph of the 4 rats studied. Since these percentages indicated that an average of 75 percent of the absorbed fatty acid was transported away from the mucosal cells via the lymphatic system, the Partition Hypothesis was considered as no longer tenable. Further evidence against the hypothesis has accumulated in the years since 1950 (4, 7, 49).

Histochemical approaches to problems of lipid absorption have historically been co-ordinate with physiological approaches; this was particularly true in those studies related to the 'form' in which lipid

was absorbed. Both Verzár (58) and Frazer (20) used histochemical evidence in support of their respective hypotheses of lipid absorption. However, much of the histochemical work was, and is, strongly criticized on the grounds of uncertainty in the specificity of the stains used (44).

The aspect of lipid absorption involving the actual means by which lipid enters the mucosal cells has scarcely been clarified by electron microscopy, though many interesting observations have been noted (43, 36, 2). Palay and Karlin (43) prepared electron micrographs of intestinal tissue taken at various postprandial time intervals from rats which had been fed corn oil. From their micrographs they observed the following: 1. Small droplets of fat (maximum diameter of 65 mμ) lodged in the intermicrovillous spaces. 2. The occasional appearance of membranous extensions of the microvilli into the terminal web. Lipid droplets could sometimes be seen enclosed within these membranous extensions - which the authors called pinocytotic vesicles, thus inferring that the absorption of the lipid was pinocytotic in nature. 3. The presence of large numbers of lipid droplets (110 to 240 mμ in diameter) in the apical portion of the mucosal cells. These droplets appeared to lie within the endoplasmic reticulum, since each droplet was enclosed by a thin membrane. 4. A noticeable accumulation of lipid within the Golgi complex, and 5. The extrusion of lipid droplets from the apical regions of the mucosal cells into the intercellular spaces. These extruded droplets were no longer enclosed by a membrane. Lacy and Taylor (36) prepared electron micrographs of intestinal tissue taken from rats fed olive oil and were able to make observations simi-

lar to those of Palay and Karlin. Lacy and Taylor, however, noted lipid inclusions within the microvilli; these were not observed by Palay and Karlin. Since electron micrographs do not allow distinctions to be made between the types of lipid observed (i.e., fatty acid vs. glyceride), the integration of the biochemical aspects of lipid absorption with the visual aspects still remains as a challenge.

The role of bile has always been an integral aspect of the interest in lipid absorption. Reports that fats were absorbed to a limited extent in the absence of bile began to appear in the literature of physiology as early as 1892. Through the years a very extensive literature has been accumulated on the relationship of bile deficiency and lipid absorption. Three papers have been selected as examples and will be briefly reviewed.

In 1935, Riegel and others (49) presented an investigation on the absorption of oleic acid from permanently isolated jejunal loops which had been prepared in four dogs. These loops were prepared by surgically resecting a small length of jejunum free from the remainder of the intestine, yet leaving the mesentary and, hence, the blood supply to the section intact. A permanent fistula through the abdominal wall was made for one end of the jejunal loop, while the other end was closed by suturing. (This is called a 'Thiry' loop and, it will be noted, is a bile-free preparation.) The Table of their data appeared as follows:

Table 1. Per Cent of Oleic Acid Not Recovered from Intestinal Loops when Introduced Alone or in Combination with Sodium Taurocholate, Hepatic Bile or Gall-bladder Bile.

MATERIALS USED	MEAN PER CENT LOSS Dog 1	Dog 2	Dog 3	Dog 4	Average all expt's.
	Removed immediately				
Oleic acid (869 mgm used in all expt's.)	5.9±0.8* (6)!	12.4±2.1 (4)	11.2±2.6 (10)	6.6±1.5 (6)	9.1±1.2 (26)
	Removed in 2 hours				
Oleic acid	15.4±1.4 (12)	10.6±2.0 (7)	12.2±6.5 (4)	10.9±1.9 (6)	12.9±3.7 (29)
Oleic acid + Na taurocholate (82-246 mgm)	45.6±2.0 (21)	36.7±3.6 (5)	21.8±1.2 (7)	28.4±3.5 (4)	38.0±2.1 (37)
Oleic acid + hepatic bile (taurocholate content: 166-275 mgm)	41.9±4.1 (5)	19.0 (1)	13.0±3.5 (5)		26.7±5.0 (11)
Oleic acid + gall-bladder bile (taurocholate content: 166-275 mgm)	35.7±2.2 (7)	23.0±3.9 (6)	16.6±2.3 (6)		25.7±2.4 (19)

* = S.E. of mean
! = Number of experiments entering into the mean.

Although general conclusions can scarcely be drawn from data involving only four experimental animals, observations arising from this study might include the following:

1. In two dogs (dogs 2 and 3) there was no indication of significant absorption of oleic acid in the absence of bile salt. In the remaining two dogs some absorption did occur in the absence of bile salt.
2. In all four dogs the presence of sodium taurocholate increased the absorption of oleic acid. This increase was not always so evident when hepatic or gall-bladder bile replaced sodium taurocholate in the infused mixture.

The authors observed that "the relative concentrations of oleic

acid and taurocholate in the fluid introduced into the loops precluded any direct chemical reaction between the two as an explanation for the absorption of oleic acid". Also, they concluded their paper with the following statement: "It is apparent, therefore, that the action of taurocholate in facilitating the absorption of oleic acid must be in the nature of a surface phenomenon and that very minute concentrations are sufficient to produce absorption of relatively large quantities of fatty acids."

Heersma and Annegers (27) presented data on lipid absorption in the absence of bile from an experimental 'balance' study. Dogs with bile diverted to the renal pelvis were fed (daily) 25 grams of lard mixed with dog food (total lipid fed was 36 grams) over a seven day period. Fecal material was collected during the last five days and a pooled fecal sample was taken for quantitative analysis of fat. Mean fecal fat output (nine dogs) in grams per day was increased from the preoperative (i.e., normal) 3gm/day level to 27.4gm/day after diversion of bile from the intestine. The following Table illustrates what happened when desicated ox bile was administered daily in an effort to decrease the steatorrhea; steatorrhea is defined as "the fecal loss of dietary fat due to impaired fat absorption".

Table 2.

Regime	Number of dogs	Average fecal fat output (grams/day)
3gms desicated ox bile	7	26
6 gms desicated ox bile	6	20

Table 2 continued.

Regime	Number of dogs	Average fecal fat output (grams/day)
6 gms desicated ox bile in solution (90 mls)	7	12.5

Two points of interest relative to the above work deserve attention.

1. Steatorrhea was associated with bile deprivation. Another study by these same authors (26) indicated that, when endogenous lipid quantities were considered, bile fistula dogs excreted about 58 per cent of the fed lipid (lard).
2. Normal lipid absorption was approached but not accomplished by rather 'casually' administered ox bile preparations - 'casual' in the sense that a). the bile salt activity may have been altered by desication and, b). the bile preparation was not administered continuously.

In 1953, Pessoa, Kim and Ivy (45) undertook a study of fat absorption in normal, in biliary-fistula and in pancreatic-duct-ligated dogs. The results from this study listed below were chosen from the data presented to illustrate differences between the normal and the bile deficient animals.

1. Endogenous fat excretion. This portion of the experiment involved an analysis of fecal fat using dogs fed fat-free diets. A total of thirty-one analyses on nine normal dogs indicated that an average of 39.4 (SD ± 1.7) mg fat/kg body weight/day were excreted. Eleven analyses on five bile fistula dogs indicated that an average of 142.2 (SD± 32.3) mg fat/kg body

weight/day were excreted.

2. Percentage absorption of corn oil. In this case, an analysis of fecal lipid was made when the diet included 2.5 ml corn oil/kg body weight. From a knowledge of fecal loss, the absorption of the lipid may be calculated from the following formula:

$$\% \text{ fat absorption} = 100 \times \frac{\text{ingested fat} - (\text{excreted fat} - \text{endogenous excretion})}{\text{ingested fat}}$$

Results indicated that five normal dogs (ten analyses) absorbed an average of 96.4 (SD ± 4) per cent of the corn oil fed, while three bile fistula dogs (three analyses) absorbed an average of 71.7 (SD ± 12.2) per cent.

3. Per cent absorption of oleic acid. In this case, an analysis of fecal lipid was made when the diet included 2.5 ml oleic acid/kg body weight. Calculations of absorption were made as explained above. Results indicated that three normal dogs (three analyses) absorbed an average of 93 (Range: 88-95) per cent of the oleic acid fed, while two bile fistula dogs (two analyses) absorbed an average of 70 (Range: 54-86) per cent.

It may be noted that the results of these three papers agree in 'quality' but not in 'quantity'; that is, all of them indicated an impaired absorption of triglyceride and/or fatty acid in the absence of bile, yet the magnitude of this impairment varied. For example, the the per cent absorption of oleic acid from the jejunal loops of dogs was negligible, while the absorption of oleic acid in bile fistula dogs (experiments of Pessoa, Kim, and Ivy) (45) averaged 70 per cent. Also, the study by Heersma and Annegers (26) indicated an average per cent

absorption of fed lard of 58 per cent, while the per cent absorption of corn oil by dogs in the Pessoa, et. al., paper (45) indicated an average value of 72 per cent. Suffice it here, perhaps, to focus attention on the quite general agreement that bile deficient animals display a reduced capacity to absorb lipid.

The recognition that bile plays a role in lipid absorption has given rise both to speculations upon the mechanism of its action and to experimentation designed to clarify its action. The experimentation has shown that bile may exert not a single action, but a multiplicity of actions. In a recent review of intestinal lipid absorption, Senior (52) presented references which implicated bile in a number of roles. One role might be termed an 'external' one in that it deals with luminal events and implicates bile in such diverse aspects of lipid absorption as emulsification, the formation of micelles, the action of pancreatic lipase, gut motility, and the reduction of fatty acid "pK_a" values. Another suggested role might, in contrast, be termed an 'internal' one in that it implicates bile in the mucosal intracellular synthesis of triglyceride. It is the postulated intracellular role of bile which will be the central theme for the remainder of this Introduction and which forms the basis for the expermental design presented in later sections. Before turning to the specific postulate of intracellular bile action, however, it would be well to look briefly at some of the 'external' roles of bile.

It may be recalled that Pflüger (46) associated bile with the "solubilization" of fatty acids and soaps, while Verzár associated bile with the formation of a fatty acid-bile salt complex which was

essential for the actual transfer of the fatty acid across the mucosal cell membrane.

Frazer, et. al. (23) found that finely emulsified paraffin oil (average particle diameter of 0.5μ) was absorbed as effectively as an olive oil emulsion of comparable particle size. Having found that 0.5μ was the average particle size of the emulsion which appeared in the intestinal lumen of rats fed olive oil, he concluded that the normally occurring emulsification system allowed the formation of particles small enough for particulate absorption. Using in vitro techniques, he found that only the triple combination of bile salts/oleic acid/monoglyceride could form an emulsion which satisfied the apparent requirements for absorption.

Hofmann and Borgström, in 1962 (28), rejected the idea that lipids were absorbed in emulsified form. Instead, they presented experimental evidence which suggested that the lipid present in the intestinal lumen was absorbed in 'micellar' form. Although some investigators have preferred the term 'micro-emulsion', Hofmann and Borgström preferred the term 'micellar solution' and attributed to it the following characteristics: "...usually transparent, have a particle size in the range of 3 to 10 mμ, form spontaneously when the concentration of amphipath (bile) exceeds the 'critical micellar concentration'...". Hofmann and Borgström postulated that the micelles formed in the intestinal lumen during the digestion of triglyceride were composed of bile salt, fatty acid, and monoglyceride. This postulate stimulated investigation and has been widely (though not entirely) accepted. Johnston and Borgström (31) have recently found that a). slices of rat intestine readily ab-

sorbed lipid when incubated in micellar solutions and b). the absorbed lipid was partially synthesized into di- and triglycerides.

Evidence was available in the early 1920's which indicated that bile activated pancreatic lipase. Borgström, who felt that the evidence was incomplete, presented a paper in 1954 (11) which showed (in vitro) that taurocholic acid (present in the concentrations most probably found in the small intestinal contents during digestion) shifted the pH optimum of pancreatic lipase from a pH of 8 to pH values between 6 and 7. These lower pH ranges were considered to be similar to those normally found in the upper intestine.

It is generally accepted that the presence of fat in the duodenum inhibits motility of the gastric antrum and slows the emptying of the stomach. Several authors, Menguy (39) and Morgan, et. al. (42) for example, found that this inhibition did not occur in the absence of bile. Menguy has concluded that the inhibitory effect was directly related to the secretion of bile which had been reflexly stimulated by the presence of fat in the duodenum. Morgan (40), however, has shown that duodenal infusions of sodium taurocholate into bile deficient rats gave no inhibition of gastric motility. Morgan also found that the inhibition occurred in bile deficient animals when emulsified oleic acid was infused slowly into the duodenum; Menguy did not find this to be true in his experiments. Morgan concluded that the inhibition of gastric motility resulting from fat in the duodenum was a function of the capacity of bile to emulsify and, hence, to promote the release of fatty acid. Menguy would not agree with this conclusion.

Let us turn now, for the remainder of the Introduction, to inves-

tigations concerned with the postulated intracellular action of bile. The paper most frequently referred to in this regard, and which is usually considered to be the 'origin' of the postulate, is a reference to experiments performed by Dawson and Isselbacher (16). Before presenting this paper, however, it would seem profitable to discuss a paper by the same authors which appeared in print a few weeks earlier (15).

This earlier paper (15) was oriented towards a delineation of the cofactors involved in mucosal glyceride synthesis. The investigators utilized the following two incubation 'systems':

1. A control system which contained Tris-maleate buffer, palmitic acid-1-C^{14}, Tween "80" (polyoxyethylene sorbitan mono-oleate; frequently used as an emulsifying agent), and an aliquot of a mucosal homogenate from which the cell walls and the nuclei had been removed by centrifugation at 600 x G.
2. A "complete" system which contained all of the above plus an aliquot of taurocholate (1.3×10^{-3}M).

Each of the above systems was incubated for 30 minutes at 37° C. in air and without agitation. A comparison of the amounts of palmitic acid-1-C^{14} incorporated into each of the glyceride fractions was presented and appeared as follows:

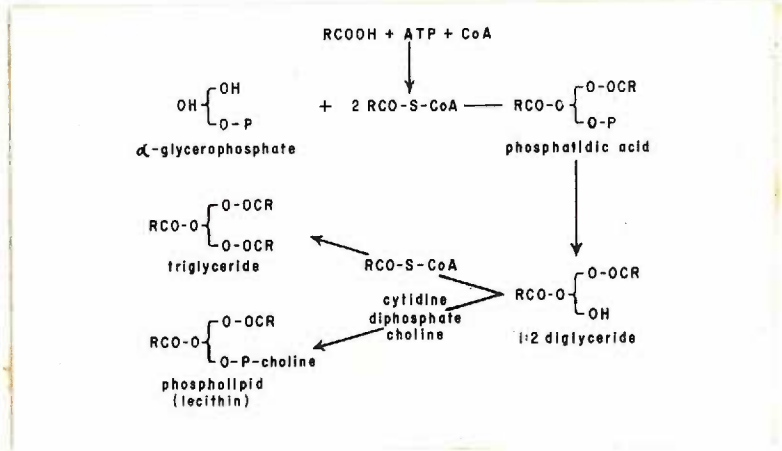
Table 3.

Incubation Mixture	Total Palmitic acid-C^{14} incorporated	Glyceride Fraction		
		Tri	Di	Mono
	μ moles	%	%	%
Control	13.2	28	49	23
Plus taurocholate (1.3×10^{-3}M)	5.2	13	22	65

The above results might infer that taurocholate had inhibited glyceride synthesis. There are two things to keep in mind, however; 1.) taurocholate (under some conditions) is considered to be a lipase activator (11,12), and 2.) the results did not indicate net synthesis - only glyceride present at the moment of extraction. The above results might, then, be explained by a lipolytic action of bile and give no indication of its possible role in synthesis.

There are many aspects of homogenate studies which cloud an attempt to assess the normal physiological role of bile. As above, many investigators use Tween "80" as a means of getting the lipid to be studied into emulsified form. This, in itself, is a complicating factor for Tween has been shown to inhibit both triglyceride lipolysis (15) and phosphatidic acid breakdown (53). This latter point, perhaps, needs some elaboration. The biochemical steps involved in the synthesis of triglyceride from absorbed fatty acid are considered to include the obligatory breakdown of phosphatidic acid to diglyceride, as illustrated in the schema shown in Figure 1. Interference with the breakdown of phosphatidic acid could be expected to alter both the total amounts and the relative amounts of the synthesized glyceride fractions.

Figure 1. (15)



Another complicating aspect of homogenate studies, when experimentally used in the investigation of bile action, is represented by the wide range of homogenate fractions possible; i.e., microsomal, mitochondrial, or supernatant. Each of these homogenate fractions has been studied in relation to glyceride synthesis and, in many cases, conflicting results have appeared. A last comment offered regarding the difficulty of interpreting results from homogenate studies relates to the problem of how much taurocholate should be added in order to mimic normal conditions. It has been found (16) that results were often dependent upon the concentration of bile salt used.

To progress, now, to investigations which deal directly with the postulated intracellular action of bile. Most of the papers to be discussed are related to whole animal preparations. The Dawson and Isselbacher paper to be discussed next, however, utilized 'intestinal slice' (1) and 'intestinal sac' (57) preparations. An intestinal slice preparation involves the incubation of a piece, or slice, of intestine in a known medium which contains a desired precursor. After incubation the slice is homogenized and the product of whatever synthesis is being studied is quantitatively extracted. The formation of an intestinal sac preparation involves the following steps: 1.) The removal and eversion of a segment of intestine, such that the mucosal side is exposed. 2.) The formation of a 'sac' by tying one end of the everted section. 3.) The insertion of a few mls incubation medium into the lumen of the sac. 4.) Tying the remaining end to form a closed sac. The sac is placed in incubation medium and, after incubation, a quantitative analysis of the fluid within the sac and/or the sac tissue itself reveals

activity which has occurred in the tissue.

Some of the problems involved in the interpretation of homogenate studies are still present in the papers to be discussed, some are removed and, as will become evident, some new problems are added.

One of the new problems added appeared in the first paragraph of the paper which will be discussed next (16). This is the Dawson and Isselbacher paper which, as mentioned before, is frequently referred to with regard to the question of intracellular bile action. "This paper", the authors wrote, "presents observations on factors influencing the uptake and esterification of palmitate-1-C^{14}." It appears pertinent to insert here the comment that, to whatever extent the processes of uptake and esterification are interrelated, so too will be the extent of the difficulty in pin-pointing the specific effect of any condition imposed upon the system.

The above investigators incubated jejunal and ileal intestinal slices with palmitic acid-1-C^{14} in a buffered solution for one hour; temperature conditions will be discussed later. To some incubation flasks they added Tween "80" as an emulsifying agent; to other flasks they added taurocholate. In those flasks with taurocholate the quantity of esterified palmitate present in the tissue was 2 to 4 times the quantity found in the tissue which was incubated with Tween "80". It was suggested that, since both taurocholate and Tween were good emulsifying agents, the difference in esterification might implicate taurocholate in a role additional to that of an emulsifying agent. It might be well to point out that the assumption that the extent of emulsification was equivalent in the above two cases was quite broad and cer-

tainly should have been checked.

The investigators utilized a 'preincubation' system in an effort to clarify the suggested "additional" role of taurocholate (the predominant bile salt in rat bile) (5, 25). This 'preincubation' system involved the following experimental steps: (Temperature conditions will be discussed later.)

1. One group of intestinal slices were preincubated for thirty minutes in a medium which contained Krebs-Ringer phosphate buffer and taurocholate (10^{-2} M). Following preincubation these same slices were again incubated for another thirty minutes in the buffered taurocholate medium to which had been added palmitic acid-1-C^{14} (100 mμ moles).
2. A second group of intestinal slices was preincubated for 30 minutes in a medium of Krebs-Ringer phosphate buffer only. Following preincubation these same slices were again incubated for another 30 minutes in Krebs-Ringer medium to which both taurocholate (10^{-2} M) and palmitic acid-1-C^{14} had been added.

The authors proposed that the concept of an intracellular role of bile would be strengthened if - during the second incubation - the intestinal slices which had been preincubated in the presence of taurocholate had synthesized more glyceride than had those slices which had been preincubated in buffer alone. In six experiments the mean palmitate esterification was 1.1 mμ moles/100 mg tissue/30 minutes in the slices which had been preincubated in buffer and 1.9 mμ moles/100 mg tissue/30 minutes in the slices which had been preincubated in the presence of taurocholate; a mean difference in esterification of +73 per

cent. This difference in esterification did not occur when Tween "80" replaced taurocholate in a similar experimental system. The Table (giving the range of experimental values) from which these data were drawn appears below.

Table 4. The Effect of Preincubation of Slices of Rat Small Intestine with Taurocholate or Tween "80" upon the Esterification of Palmitate-1-C^{14}.

Addition	No. of Expt.	Palmitate esterification/100 mg tissue after preincubation with: Buffer only Mean	Range	Buffer + wetting agent Mean	Range	Mean change in palmitate esterification
		mμ moles		mμ moles		%
Taurocholate (10^{-2} M)	6	1.1	0.7-1.5	1.9	1.0-2.4	+71
Tween "80" (0.1%)	4	0.43	0.28-.60	0.40	0.24-.61	-7

With regard to the experimental data presented above, it should be mentioned that an increase in mucosal synthetic ability is not the only possible explanation for the observed increase in glyceride synthesis in the tissue preincubated with taurocholate. It is possible that the prolonged presence of taurocholate altered mucosal permeability. If, for example, the prolonged presence of taurocholate increased the permeability of the mucosal cells to fatty acid, the amount of glyceride synthesized might well be increased.

In all of the above work the authors have assessed net glyceride synthesis by extraction of glyceride from the tissue. Ordinarily, to arrive at figures for net synthesis, it would be necessary to analyze the incubation medium for glyceride which had escaped from the mucosal

cell. The authors eliminated this necessity by incubating the slices at a temperature of 25°C. instead of 37°C. At this lower temperature they had previously found that the escape of glyceride from the tissue was reduced to almost zero. It may be wondered whether synthesis was 'normal' under these conditions.

In an attempt to determine whether the observed increase in glyceride synthesis in the presence of taurocholate depended upon an increase in absorption of the labeled fatty acids or to an increase in triglyceride synthetic ablility, the authors utilized uniformly labeled glucose-C^{14} in the following experiment: Everted rat small intestine was incubated in a buffer solution containing 0.02 μ moles (5×10^5 epm) of the labeled glucose. After an incubation period of one hour the tissue was homogenized and the tissue lipid was extracted. It was found that, if taurocholate was added to the medium, the extracted lipid contained four times as much label as did lipid extracted from tissue incubated with glucose-C^{14} in the absence of taurocholate. When the extracted lipid was saponified, more than 95 per cent of the label appeared in the water-soluble fraction; from this it was concluded that the radioactivity was present as labeled glycerol. Since no fatty acid was present in the medium, the observed increase in label incorporated into lipid in the presence of taurocholate was attributed by the authors to a "stimulated lipid metabolism in the mucosal cell". The actual data are given in the second column of the following Table: (Each result is a mean of triplicate determinations).

Table 5.

Addition	Radioactivity/100 mg tissue	
	Total lipid (cpm)	Barium-ethanol ppt. (cpm)
None	4,160	3,430
Taurocholate (2×10^{-2} M)	16,400	3,360

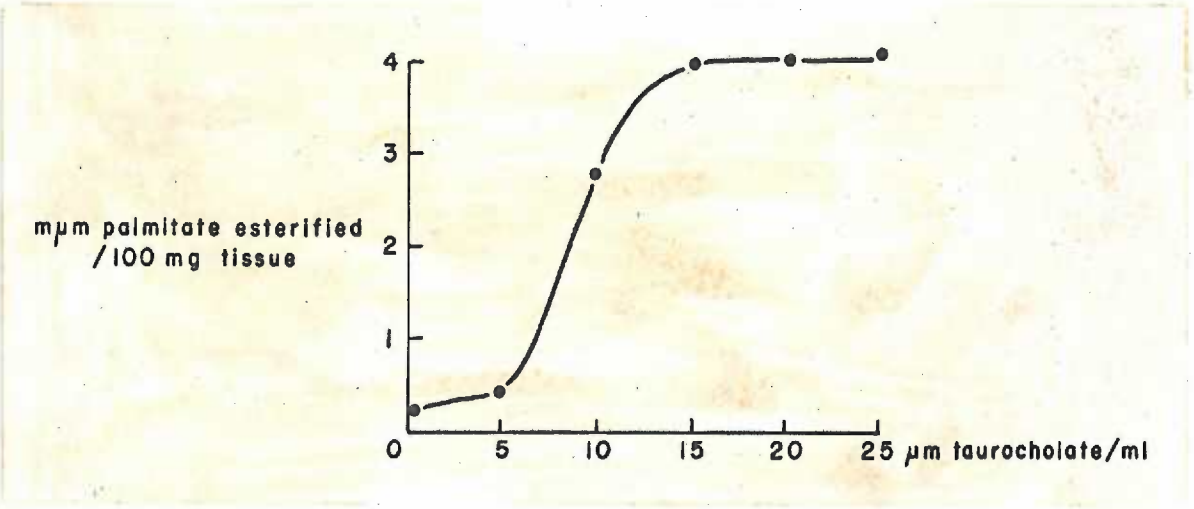
It may be noted that the actual maximum incorporation of glucose-C^{14} (i.e., in the presence of taurocholate) amounted to only 0.00066 μ moles/100 mg tissue. The above results might have occurred if the conjugated bile salt had increased the labeling of the intracellular glucose pool, secondary to a greater transport of the tracer glucose into the cell. The authors considered that, if this were the explanation, an increase in the labeling of other products of glucose metabolism (such as phosphorylated carbohydrate intermediates) should have occurred. The phosphorylated carbohydrate intermediates were therefore isolated by barium-ethanol precipitation and (as shown in the third column of the above Table) did not show any increase in radioactivity when taurocholate was present in the incubation media.

It is difficult to draw conclusions from the experiment discussed above. It would seem that the very small quantities of substances involved in this situation of 'dynamic' metabolism might not necessarily reflect a situation which involved the absorption of a fatty acid and the production of relatively large amounts of glyceride.

It appears pertinent, at this point, to draw attention to evidence which has indicated that the concentration of bile salts with which one works makes a very significant difference in the experimental results.

Figure 2 illustrates the effect of varying concentrations of taurocholate on glyceride synthesis. Standard incubation procedure was used. There were 100 m μ moles of palmitate-C^{14} in each flask and taurocholate was added as indicated.

Figure 2. (16)



At 5 μ moles/ml or less there was no apparent effect and maximum effects were found at 15 μ moles/ml or more. The authors usually used 'optimum' concentrations (i.e., 20 μ moles/ml) of taurocholate in their experiments; an exception appeared in the experiments of Table 4, wherein the authors used 10 μ moles/ml taurocholate. In vivo experiments reported by Danielsson (14) indicated that the main absorption of lipid occurred at a bile salt concentration of 5 to 10 μ moles/ml.

To summarize this paper presented by Dawson and Isselbacher it might be appropriate to suggest that:

1. If the assumption is correct that Tween "80" and taurocholate emulsify equally (in the concentration used) and, also, that Tween "80" is otherwise 'inert', then it would appear that tauro-

cholate has a role in lipid absorption which is additional to its function as an emulsifier.

2. If the assumption is correct that there are no alternative explanations for the observed increase in triglyceride synthesis in tissue slices which had been preincubated with taurocholate, then it would again appear as though taurocholate plays a role in lipid absorption which is additional to its function as an emulsifying agent.
3. Although there is some evidence that taurocholate affected mucosal intracellular metabolism, this same evidence does not necessarily infer an identical effect upon net glyceride synthesis.

Before turning to another paper, it is well to note that the authors were aware of the fact that the action of taurocholate in the homogenate system of their earlier paper (15) was different from its action with the tissue slices in their second paper (16). This second paper, which has just been discussed, ends with the following statements: "The difference between the results using these two techniques probably is due to the abnormal structural relationships of cellular particles in the homogenate system. Thus, although homogenates are of use in delineating reactions and their cofactor requirements, the use of intact cells is mandatory to evaluate the possible physiological role of such reactions."

Attention will now be directed to a paper presented by Borgström in 1953 (10). Actually, it was this paper which provided the initial suggestion that bile exerts an affect upon mucosal metabolism. Concerted investigation upon the problem, however, seems to have waited until its

're-introduction' by Dawson and Isselbacher.

Borgström prepared four bile deficient rats by cannulating the bile ducts and draining the bile to the outside. In these same four rats he installed cannulae into the main intestinal lymphatic ducts. The day after the surgical procedures the rats were fed a meal (by stomach tube) of corn oil[1] carrying 0.5 per cent dissolved, free palmitic acid-1-C^{14}. Borgström then collected the lymph in periods of 0-2, 2-4, 4-6, 6-9, 9-12, and 12-24 hours after feeding. 24 hours after feeding he sacrificed the animals and analyzed the gastrointestinal tract for remaining activity.

Results were as follows: These four experimental rats, in which both intestinal lymphatic ducts and bile ducts were cannulated, absorbed (in 24 hours) a mean of 65 per cent of the label fed (the range included 91.7, 84.2, 43.8, and 41.9 per cent)[2]. A mean of 16.9 per cent of the absorbed activity appeared in the intestinal lymph lipid. In a previous experiment, Borgström (9) had fed the same lipid mixture to normal rats with lymphatic cannulae and had found that 87.5 per cent of the label was absorbed and, of this, 66.5 per cent was recovered in lymph lipid.

An interesting aspect of this experiment focused attention on bile as it appeared to affect the synthesis of glyceride within the mucosal cell, and/or, the transport of the labeled lipid from the mucosal cell into the lymphatic system. This aspect was associated with the difference

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1. 0.1 ml lipid per square dm body surface.

2. This insertion of a range of values illustrates oneof the most difficult facets of investigations on parameters of 'absorption' - the great range of variability between animals.

in the ratio of $\frac{\text{percentage of label absorbed}}{\text{percentage of label recovered in lymph}}$ between the bile deficient and the normal rats. In bile deficient rats the ratio of means appeared to be 65/16.9 or 3.8; while in normal rats it appeared to be 87.5/66.5 or 1.3.

Within the limitations imposed by the small number of animals used, the following might be said in summary of the Borgström paper: The data presented indicated that in the bile deficient animals there was not only an impairment of absorption of lipid, but also an impairment in either the synthesis of triglyceride (assuming this to be requisite for lymphatic transport) or the removal of the label from the mucosal cell into the lymph.

An experimental design somewhat similar to that used by Borgström was presented by Saunders and Dawson (50). They fed labeled oleic acid (either in unemulsified form or as an aqueous emulsion) to the following two groups of rats: 1. A control group bearing only thoracic duct fistulae and, 2. An experimental group bearing both thoracic duct and bile fistulae. Lymph was collected for 12 hours after feeding in both groups. All rats were sacrificed after this 12 hour period. The gastrointestinal tracts (with feces) were removed at sacrifice and the lipid extracted.

When 80 mg unemulsified uniformly labeled oleic acid-C^{14} was fed via stomach tube to control rats a mean (11 animals) of 72 per cent of the fed radioactivity was absorbed in the 12 hour postprandial period, 81 per cent of the absorbed amount appeared in the lymph, and 5.6 per cent of the label in the lymph was in the form of free fatty acid. There appeared to be no difference in results if the oleic acid-C^{14} was given by duodenostomy rather than by stomach tube, or if it were given

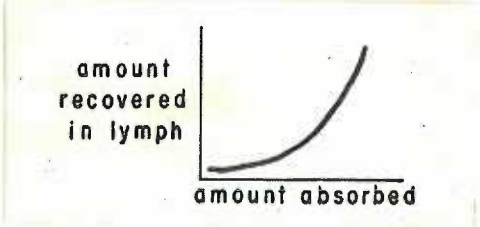
by duodenostomy as an emulsion in Tween "20" rather than in unemulsified form. When unemulsified uniformly labeled oleic acid-C^{14} was given to the rats with bile fistulae a mean of 49.1 per cent of the fed radioactivity was absorbed, 7.2 per cent of the absorbed label was recovered in the lymph, and 28 per cent of the label in the lymph was in the form of free fatty acid. There was no significant difference between the volumes of lymph produced by bile deficient and control rats. In terms of actual amounts the above percentages appear as follows:

Table 6.

Control Animals		Bile Deficient Animals	
1. Fed	80.0 mg	1. Fed	80.0 mg
2. Absorbed	57.6 "	2. Absorbed	39.3 "
3. Appeared in lymph	46.7 "	3. Appeared in lymph	2.8 "
4. As neutral lipid in lymph	44.1 "	4. As neutral lipid in lymph	2.03 "
5. As free fatty acid in lymph	2.6 "	5. As free fatty acid in lymph	.79 "

As in Borgström's paper (10), a consideration of the ratio $\frac{\text{mg lipid absorbed}}{\text{mg lipid recovered in lymph}}$ in control vs. bile deficient animals tempts one to infer an inferior synthesis of glyceride in the absence of bile. The following alternative inference might, however, be suggested: The decrease in absorption in the bile deficient cases may retard the removal of lipid from the cell out of proportion to the decrease in absorption itself. In other words, the amount of lipid removed from the mucosal cell may not always be linearly related to the amount of lipid absorbed but might, perhaps, follow a relationship such as shown in Figure 3.

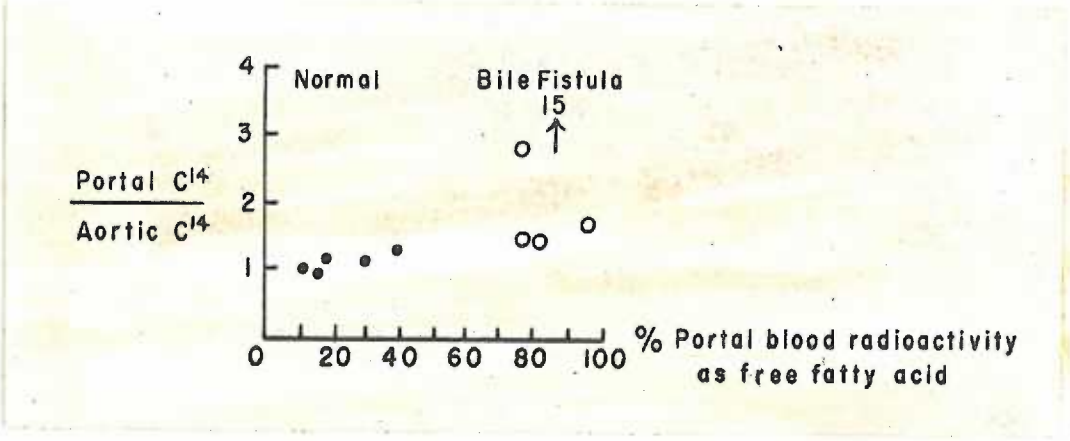
Figure 3.



Such an alternative might be the reason why, in a later experiment (50), control animals fed only 8 mg oleic acid-C^{14} absorbed 87 per cent of the fed amount, but only 63 per cent of the amount absorbed was recovered in the lymph. It seems to be a case of trying to assess what goes on inside a 'black box' by knowing the input and the output; one problem, however, is that the 'black box' contains two variables - synthesis and removal. The crucial investigation here would seem to be an analysis of the mucosa itself and the relationships between the amounts and forms of the lipids contained within it, in both normal and bile deficient animals. These workers abandoned a mucosal project apparently, having found that only 2 to 3 per cent of the fed lipid could be recovered from the entire intestinal wall.

Saunders and Dawson (50) also presented an experiment in which they examined the ratio of C^{14} present in portal vs. aortic blood in both bile deficient and normal rats. The blood samples were taken ½ to 2 hours after feeding oleic acid-C^{14}. The ratio of C^{14} in portal blood to C^{14} in aortic blood in bile fistula animals appeared to be slightly higher than the ratio in normal animals, but it is doubtful if a significant difference could be shown (ratios are indicated below in Figure 4). The per cent portal blood C^{14} present as fatty acid was, however, significantly higher in the bile fistula animals than in the control animals (75-95% and 10-40%, respectively); as indicated in Figure 4. The authors suggested that bile may exert an effect either upon synthesis or, perhaps, upon the route which the absorbed lipid follows in its removal from the cell.

Figure 4. (51)



In further investigations Saunders and Dawson (50) found that when bile deficient rats were fed oleic acid-C^{14} which had been emulsified with either Tween "20" or taurocholate - rather than unemulsified oleic acid-C^{14} several differences could be observed. The absorption of the oleic acid-C^{14} rose to a mean of 57.6% with the Tween "20" emulsion and to a mean of 66.5% with the taurocholate emulsion. Also, the mean percentage of absorbed oleic acid-C^{14} recovered in the lymph was 44.5% with the Tween "20" emulsion and 50.7% with the taurocholate emulsion and, while taurocholate reduced the percentage of lymph lipid present as free fatty acid - Tween "20" did not. When 8 mg sonicated oleic acid-C^{14} (prepared by emulsifying the fatty acid in an ultrasonic disintegrator for 1 hour; the optical density of this emulsion was 1.09) were fed to bile fistula animals the absorption of the lipid did not appear to have significantly increased above the average level for bile deficient animals (i.e., 47%), nor did the percentage of lymph lipid present as free fatty acid appear to be altered (i.e., 23%); the recovery in the lymph of the absorbed C^{14} was, however, raised to 72%. From this collection of experimental data the authors concluded the

following: 1. Bile facilitated glyceride synthesis, as indicated by the increased percentage of fatty acid in the lymph in the absence of bile and, 2. Bile, in its capacity as an emulsifier, altered the pathway by which lipid in the mucosal cell was removed (as indicated by recovery of absorbed lipid in the lymph).

The experiments relating to recoveries of fatty acid-C^{14} in the blood do not appear quantitative enough to warrant a statement that bile does, or does not, determine the pathway of lipid removal from the mucosal cell. It would seem, however, that such a possibility warrants further investigation. It is well to mention, here, that blood samples were taken under ether anesthesia; a procedure which has been reported (6) to have profound effects on lipid absorption.

One small point before closing a discussion of this paper; the sodium taurocholate used in this paper contained "less than 5% free cholic acid". A quotation from Dawson and Isselbacher (16) seems pertinent here. "Investigations on the physiology of absorption when crude bile salts are used must be interpreted with caution. Unless stringent tests of purity are employed the results may be misleading, for traces of unconjugated bile salts (especially desoxycholate) can alter mucosal function".

A rather complicated but unique experiment has recently been reported by Morgan (41). It seems appropriate to discuss this paper next because it tends to support the evidence (as presented above) that the particle size of the lipid available for absorption may strongly influence events occurring within the mucosal cells. In brief, her experiment was as follows: A group of control rats had thoracic duct cannulae and

duodenostomy tubes installed. Another group of 20 rats had cannulae installed in the bile ducts, as well as thoracic duct fistulae and duodenostomy tubes. Forty-eight hours postoperatively she began experimental procedures which consisted of the following: 1. A slow infusion of small quantities of lipid at a constant rate through the duodenostomy tube. The infusion period lasted 10 hours, but was divided into two 5 hour periods. 2. During the first 5 hour period on the first day of experimentation the rats received a lipid mixture which had bile added. During the second five hour period the rats received a lipid mixture to which no bile had been added. 3. On the second day of the experiment the order of infusion was reversed, that is, the rats received a lipid mixture which contained no bile during the first 5 hours and a lipid mixture which contained bile during the second 5 hours. Thus, each animal was its own control - in the sense that measurements of lipid absorption were made in the same animal with and without the presence of bile. A summary of results relevant to the bile fistulae rats indicated that: 1. When the lipid administered was unemulsified oleic acid the addition of bile to the infusate increased the amount of esterified lipid appearing in the lymph and, 2. When the lipid administered was emulsified oleic acid[3] the addition of bile to the infusate appeared to have no effect upon the quantity of esterified lipid recovered in the lymph. This quantity appeared to stabilize at around 80% of the amount fed and was only slightly less than that recovered in normal animals.

The Saunders and Dawson paper (50) and the Morgan paper (41), which

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3. An Upjohn product; emulsified with lecithin and oxyethylene oxypropylene polymer.

have just been discussed, strongly suggest that the increase in the appearance of lipid in the lymph of animals which have been fed oleic acid when bile is present is a function of the capacity of bile to emulsify. Whether the presence of emulsified oleic acid within the cell determines the pathway the absorbed lipid will finally take or whether it allows a more rapid enzymatically catalized glyceride synthesis is uncertain. The Dawson and Isselbacher paper (16) (reviewed on pages 17 - 23 of this Introduction) does not support the suggestion.

Before going on to continued work, it might be well to pause and comment upon the experimental methods used in the papers discussed so far. One comment might be upon the use of Tween as an emulsifier; this, it may be recalled, has been subject to question in previous discussions. Another comment concerns procedures involving lymphatic drainage: It is unknown to what extent the removal of lymph may alter absorption, yet it seems reasonable to suggest that if circulatory effects are at all pronounced due to removal of protein by lymph drainage, the secondary effects upon absorption might be quite considerable. This comment is specifically mentioned with regard to the Morgan paper (41); her last experiments were run 80 hours after lymphatic cannulation. The problem of surgical trauma and its effect upon absorption is also pertinent, although it is somewhat diminished by the use of controls which have had almost equally extensive surgery.

Morgan and Simmonds (42) approached the problem with a novel experimental design. They proposed to study the extent of lipid (coconut oil commercially emulsified with glycerol monostearate and Tween) absorption in two groups of rats; one group with bile diverted to the

terminal ileum and one group with bile diverted to the urinary bladder. The theory here was as follows: Bile salts are known (3) to be well absorbed in the ileum. These absorbed bile salts circulate in the blood, are removed by the liver, and are resecreted into hepatic bile. The group of animals with bile diverted to the ileum might, therefore, be assumed to have relatively normal levels of bile salt in the blood and tissue fluids. Any difference in lipid absorption between this group of rats and the group with total bile deprivation (bile diversion to bladder) might be considered to represent an "intracellular" action of bile - or at least an action quite separate from its emulsification capacity. Animals in both groups were sacrificed 3 hours after the coconut oil emulsion was fed. At this time the entire gastrointestinal system was removed and the lipid remaining in it was assessed. The amount of lipid absorbed was calculated by subtracting the total lipid recovered from the total amount fed. Results were as follows:

Table 7.

	Lipid Absorbed
Control Rats:	
Group 1. Normal	170.8 mg
Group 2. Bile to duodenum (sham)	150.1 "
Group 3. Blind ileal cannula (sham)	122.2 "
Experimental Rats:	
Group 4. Bile to ileum	107.1 mg
Group 5. Bile to urinary bladder	65.0 "

With allowances made for endogenous fat (previously experimentally measured), the authors found a significant difference between group 5 and all other groups and between group 4 and groups 1 and 2. In summary, then, the above data indicated that presumably normal blood and tissue levels of bile salt (group 4) partially repaired a defect in lipid

absorption due to exclusion of bile from the lumen. The question becomes, was an intracellular action of bile necessarily involved? To discuss this question it would seem best to limit the use of the phrase "intracellular action" such that it denotes 'action' upon any one or all of the events which concern the absorbed lipid once it is inside the mucosal cells, and not upon mucosal permeability. With this narrowing of definition, let us return to the above data.

If the greater absorption of lipid which occurred when bile was diverted to the ileum (compared to diversion of bile to the bladder) occurred because of an increased permeability to the lipid resulting from the possible 'resecretion' of bile salts from the intestinal wall into the intestinal lumen, the effect would not (in accordance with the above suggested limited definition) be considered to be the result of "intracellular bile action". Forbes and Lines (18) have shown (with rats) that, though bile had been shunted to the caecum, quite considerable amounts of bile salt still appeared in the intestinal lumen. This apparently resulted from a process of 'resecretion' and was evident after ten days from the operative procedure. Since Morgan and Simmonds used rats experimentally 7 - 10 days postoperatively, the above comments may be only tangentially pertinent. It is perhaps appropriate to add, as an aside, that Simmonds and Morgan found no evidence of 'regurgitation' (back-flow) of bile from the ileum into the more proximal regions of the intestine.

To use the data from the Morgan and Simmonds paper (42) to implicate bile in "intracellular action" in the previously discussed limited sense, one would have to assume that the removal of lipid from the lumen

was directly related to glyceride synthesis and/or removal of lipid from the mucosal cell. Regarding this latter aspect, the experiment might have been improved had lymph been drained and calculations made for assessing the relationship between lipid absorbed and lipid recovered.

One possible alternative yet to be mentioned in the interpretation of data from the Morgan and Simmonds paper is related to gastric emptying and intestinal motility. If, in group 5 of Table 7, the stomach failed to release the lipid meal into the intestine or if the intestinal motility was found to be very rapid, the poor absorption would be readily explained. The authors tested both alternatives and found neither to be true.

Several paragraphs ago a reference was given which indicated that bile salts were well absorbed in the ileum (3). This reference (and others) indicated that, not only was bile salt well absorbed in the ileum, it was absorbed much better there than in any other portion of the gut; in fact, absorption increased almost exponentially with increasing distance from the pylorus of the stomach (3, 54). This is in contrast to data concerning the site of maximal fatty acid absorption. Johnston (30) found that the uptake of palmitic acid-C^{14} by hamster jejunal sacs was 3 times that of distal ileal sacs. The combination of these two geographical aspects of absorption does not eliminate a proposed intracellular action of bile - yet it certainly remains of interest to the question.

The last paper to be reviewed in this Introduction has been selected to terminate the discussion of a proposed intracellular action

of bile because it utilized a technique which has been pursued in the experimental design of this Thesis. The authors, Knoebel and Ryan (33), prepared bile fistula dogs by diverting the bile to the renal pelvis of one kidney (cholecystonephrostomy). The operated dogs were allowed to recover from the surgery for 8 to 10 days prior to use in an experiment. On the day of the experiment both normal and bile fistula dogs were fed a meal which consisted of 25 gm oleic acid, 25 gm casein, 25 gm sucrose, and 5 gm cellulose flour. In one set of experiments, not to be discussed here, the 25 gm oleic acid was replaced by 25 gm cottonseed oil. The dogs were sacrificed 4 hours after feeding and the contents of the stomach and small intestine were removed separately and saved. The intestine was divided into two portions (duodenal-jejunal and ileal), each portion was split longitudinally and the mucosal surface washed with both distilled and tap water[4], and finally, the mucosa was collected by scraping the mucosal surface of the intestine with a spatula. The lipids present in the gastrointestinal content and in the mucosa were extracted with a lipid solvent system and the extracted lipids were separated into component fractions.

A summary of the data on mucosal lipids appears in Table 8 below. The values presented have been corrected for endogenous lipid; that is, the weight of the lipid fractions found in the mucosal samples of fasted animals has been subtracted from the weight of the lipid fractions found in the mucosal samples of fed animals. Both Table 8 and Table 9 present mean values calculated from the data of 7 - 8 dogs.

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4. My own histological work has indicated that washing mucosa with distilled water must be avoided if normal mucosal histology is to be preserved.

Table 8.

	Duodenum-jejunum		Ileum	
	Normal	Bile deficient	Normal	Bile def.
Lipid weight (mg/100 gm wet mucosa)	289.0	161.0	441.0	223.0
Free fatty acid (as % of lipid wt.)	19.6	27.4	11.5	19.7
Monoglyceride (as % of lipid wt.)	5.2	12.4	3.9	11.6
Diglyceride + Triglyceride (as % of lipid wt.)	75.2	60.2	84.6	68.7

The authors concluded that their data supported the concept that intracellular esterification is retarded in the absence of bile, since a greater than normal percentage of the mucosal lipid of bile deficient dogs appeared in the form of free fatty acid (i.e., 27.4 vs. 19.6 and 19.7 vs. 11.5).

From a more detailed Table (Table 9) of the data presented by Knoebel and Ryan, it is interesting to note the following:

1. The increase in mg fatty acid in the fed vs. the fasting condition is very similar in both control and bile deficient dogs (duodenal-jejunal; 57 to 114 mg, and ileal; 62 to 113 mg in control dogs: duodenal-jejunal; 55-99 mg, and ileal; 67-111 mg in bile deficient dogs).
2. The decreased amount of mucosal lipid in fed bile deficient dogs vs. fed control dogs (duodenal-jejunal; 393 vs. 567 mg, and ileal: 490 vs. 719 mg) is largely a reflection of decreased amounts of synthesized di- or triglyceride in the bile deficient dogs (97 and 153 mg in bile deficient dogs vs. 217 and 373 mg in control dogs).

Table 9.

	Normal duodenum-jejunum		Bile deficient duodenum-jejun.		Normal ileum		Bile-def. Ileum	
	fat-free diet	oleic acid added	fat-free diet	oleic acid added	fat-free diet	oleic acid added	fat-free diet	oleic acid added
	mgs	mgs	mgs	mgs	mgs	mgs	mgs	mgs
Free fatty acid + glycerides	278±17	567±39	232±18	393±45	278±16	719±88	267±57	490±43
Free fatty acid	57±6	114±7	55±4	99±22	62±6	113±13	67±10	111±12
Monoglyceride	20±2	35±3	14±1	34±10	20±2	37±8	16±2	42±11
Diglyceride + Triglyceride	201±13	418±41	163±17	260±21	196±13	569±76	184±47	337±29

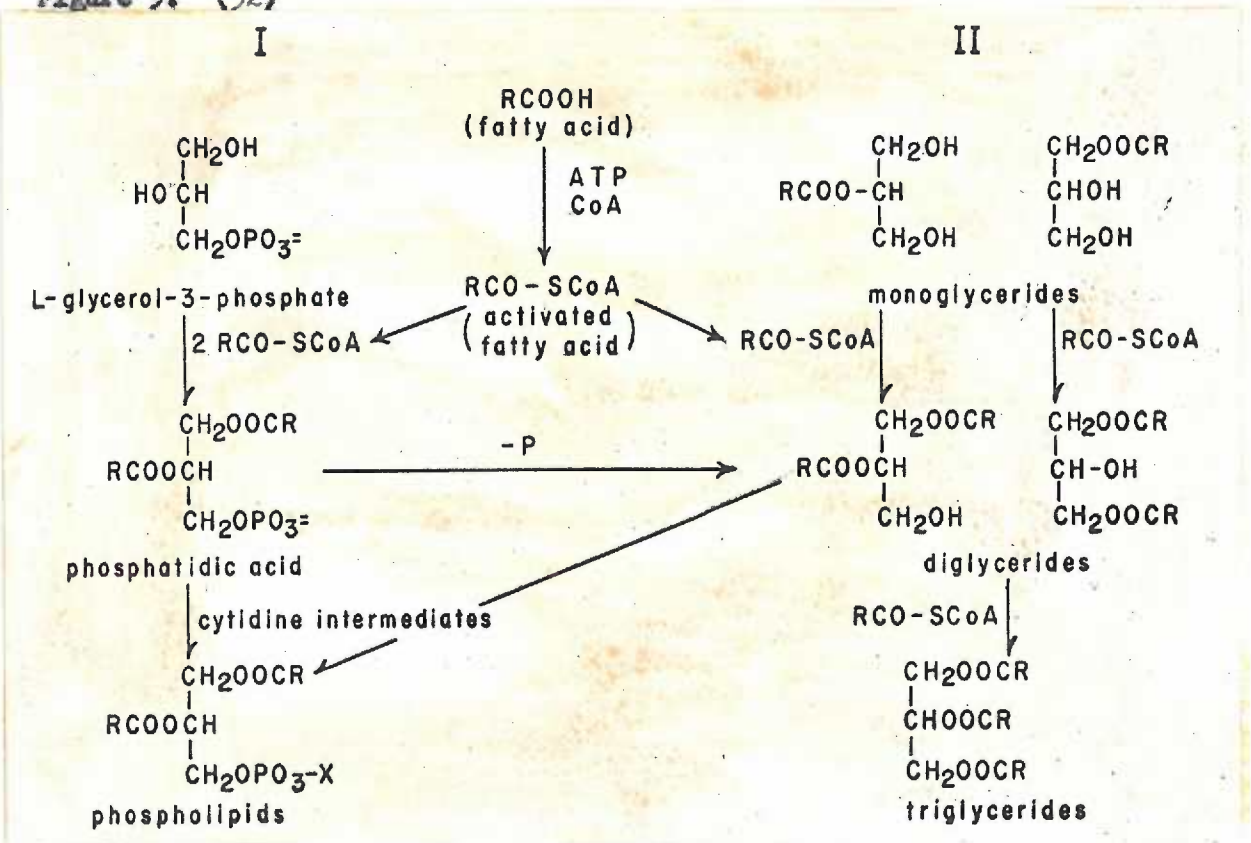
In conclusion to this Introduction and Review of the literature, it must be stated that the critical comments which have been applied to each of the papers discussed do not imply that the experimental designs have been poorly conceived. They may, instead, be attributed to the complexity of the problem and serve to indicate that most experimental results are subject to alternative explanations.

METHODS AND MATERIALS

A. Experimental Design.

As was discussed in the Introduction, the assessment of a postulated intracellular action of bile has been equivocal because of the difficulty in separating the parameters of mucosal lipid metabolism; i.e., parameters involved with the transfer of lipid from the intestinal lumen into the mucosal cells, the intracellular synthesis of triglyceride, and the removal of the synthesized lipid from the mucosal cells. The experimental design to be discussed in this section was considered as a means of looking directly at the triglyceride synthesis parameter. Before discussing the experimental design, however, it would seem well to consider the proposed schema of the biochemical events involved in the mucosal synthesis of triglyceride.

Figure 5. (52)



It will be noted that two alternative pathways of diglyceride synthesis appear in Figure 5. The pathway designated by numeral II has been termed the "direct monoglyceride pathway". The pathway designated by numeral I has been termed the "glycerol phosphate-phosphatidic acid pathway". Definitive evidence of the presence in rabbit mucosal mitochondria of the enzymes requisite for these two pathways was presented in 1961 by Clark and Hubscher (13). Circumstances probably determine which of these pathways is quantitatively the most important. It will be noted that the glycerol phosphate-phosphatidic acid pathway is obligatory when the absorbed lipid is fatty acid. If the absorbed lipid includes fatty acids and monoglycerides the monoglyceride pathway may be preferential, for Holt, Haessler and Isselbacher (in preparation, 1964), as quoted by Senior (52), found that "the addition of monoglycerides to the medium in which fatty acids and glucose or glycerol are being synthesized into higher glycerides very markedly reduces the incorporation of the carbohydrate into glyceride glycerol."

It may also be noted that the absorbed fatty acid is 'activated' to a fatty acid-coenzyme A complex (RCO-SCoA) before it enters either pathway of triglyceride synthesis. This consideration is based on the following chain of evidence: 1. In 1952 Kornberg and Pricer (34) reported that the enzymatic (a "soluble enzyme" system prepared from the supernatant fraction of a liver homogenate) esterification of L-a-glycerophosphate by long carbon-chain fatty acids to produce phosphatidic acid required the participation of ATP and coenzyme A. 2. In 1953 these same authors (35) isolated (using the same enzyme system) a fatty acid-coenzyme A intermediate. 3. Mitochondrial enzyme

preparations (from mucosal cells), as used by Clark and Hübscher (13), required the presence of ATP and coenzyme A in the incubation medium for the synthesis of di- and triglyceride from palmitic acid-C^{14}. By analogy with the liver enzyme systems, the fatty acid-coenzyme A intermediate was postulated to be an intermediate in mucosal glyceride synthesis.

With the above brief presentation of the postulated biochemical events occurring in glyceride synthesis, let us continue on to a discussion of the experimental design utilized in this Thesis. This discussion of the experimental design will consist of the following four parts: 1. A statement of the problem to be solved and a proposal for its solution. 2. A justification of the proposal in terms of its potential value. 3. A consideration of two inherent aspects of the proposal. 4. The equational analysis involved in the proposal.

1. <u>A statement of the problem and a proposal for it solution</u>.

The problem: Is bile involved as a catalyst in mucosal triglyceride synthesis?

A Proposal for the Solution of the Problem: The mass law states that "the rate of a chemical reaction is proportional to the active masses (concentrations) of the reactants". If both the rate of reaction and the active concentrations(s) of the reactant(s) involved may be measured, it becomes possible to establish the proportionality constant which relates the two. Such a proportionality constant indicates, in a sense, the relative ease with which a precursor is converted to a product. The experimental design utilized in this Thesis involves an attempt to evaluate the

proportionality constant characteristic of mucosal triglyceride synthesis during active absorption of fatty acid.

2. Potential value of the proposal.

If the proposed analysis would allow the determination of the proportionality constant characteristic of mucosal triglyceride synthesis, a powerful tool would be provided which could be used in the resolution of the problem concerning the postulated intracellular role of bile. Thus, if the proportionality constant as determined for normal rats was found to be different from that determined for bile deficient rats, it might be stated that the presence of bile influenced the intracellular synthetic process. If, in contrast, the proportionality constant as determined for mucosal triglyceride synthesis was found to be similar in both normal and bile deficient rats, it might be stated that bile exerted no influence upon the intracellular synthetic process.

It is well recognized that such a proportionality constant is gross in the sense that its magnitude is a function of all the events occurring in the precursor-product pathway. Yet, it seems reasonable to suggest that a resolution of the question of intracellular action of bile must finally come from a comparison of quantitative measurements (at the cellular level) of the parameters involved in triglyceride synthesis in normal vs. bile deficient animals.

If bile exerts a catalytic effect upon mucosal triglyceride synthesis, such an effect would represent a major contribution to the process of lipid absorption. This contribution was implied

in the following quotation from a review article by Isselbacher and Senior (29): "Since 1880, it has been known that fatty acids fed into the intestinal lumen appear mainly as triglycerides in lymph. Therefore, it is evident that one of the major functions of the small bowel is the resynthesis of triglycerides.....

3. Two inherent aspects of the proposal.

The equational analysis designed to determine the proportionality constant characteristic of mucosal triglyceride synthesis is based upon a set of assumptions and conjectures. If, for any reason, the assumptions or conjectures are invalid, the equational analysis becomes invalid. The data to be accumulated for the use of the proposed equations must also be the data upon which the validity of the proposed analysis is based. The experimental results of this investigation must, then, be considered from the following two viewpoints:

a). Is it possible to accumulate data which, when used in context with the equational analysis, gives meaningful proportionality constant values?

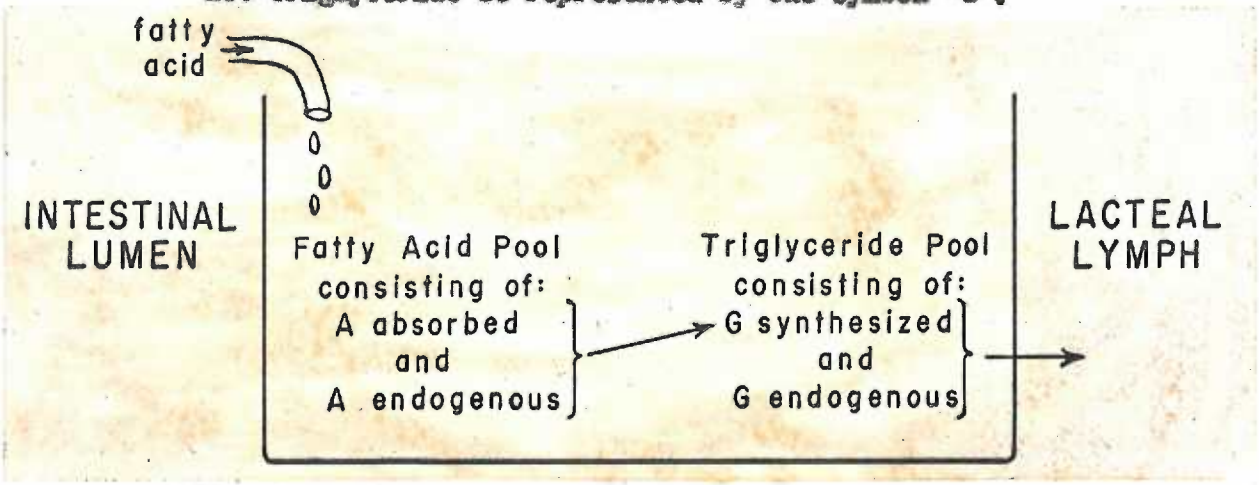
b). If the proportionality constant values are not meaningful, may the accumulated data be used in the re-evaluation of the assumptions and conjectures used in the equational analysis? Such a re-evaluation, though not the central theme of this Thesis, would contribute some insight into the events associated with in vivo mucosal triglyceride synthesis. Data representing such events is insufficiently, if at all, available in the literature.

4. The equational analysis involved in the proposal.

The equational analysis was based upon the following proposed 'system':

Figure 6.

Let the following 'compartment' represent 1 gram of mucosa.
Let fatty acid be represented by the symbol 'A'.
Let triglyceride be represented by the symbol 'G'.



The 'input' represents the absorption of fatty acid from the intestinal lumen into the mucosal cell. Both fatty acid and triglyceride pools are represented as indicated. The following considerations, based on the above, would seem reasonable:

1). A change per unit time in triglyceride concentration within the compartment will be a function of the triglyceride synthesized per unit time and the triglyceride removed per unit time, or,

dG/dt net = triglyceride synthesized per unit time minus (-) triglyceride removed per unit time; where 'G' denotes mg triglyceride/gm mucosa.

2). The amount of triglyceride synthesized per unit time will be a function of the size of the fatty acid pool, or,

dG/dt synthesized $\sim$ A = $K_1 A$; where 'A' denotes mg fatty acid/gm

mucosa.

3). The amount of triglyceride removed per unit time will be a function of the size of the triglyceride pool, or,

dG/dt removed $\propto$ G = K_2G

4). dG/dt net = $K_1A - K_2G$

Since the above expression for dG/dt net involves two unknowns, a second equation must be designed so that a simultaneous solution is possible. If the fatty acid input carries labeled fatty acid, the following considerations may be made:

1). dG*/dt net = dG*/dt synthesized - dG*/dt removed; where 'G*' denotes the radioactivity (in dpm) present in 'G '.

2). The amount of label appearing per unit time in the triglyceride synthesized will be a function of the amount of triglyceride synthesized and the specific activity of the precursor (fatty acid) pool, or,

dG*/dt synthesized = K_1AS_A, where 'S_A' denotes the specific activity of the fatty acid pool (dpm/mg).

3). The amount of label removed per unit time will be a function of the amount of triglyceride removed and the specific activity of the triglyceride pool, or,

dG*/dt removed = K_2GS_G where 'S_G' denotes the specific activity of the triglyceride pool (dpm/mg).

Now there are two equations involving the relationships of net triglyceride, triglyceride synthesized and triglyceride removed:

1). dG/dt net = $K_1A - K_2G$ (equation 1)

2). $dG^*/dt \text{ net} = K_1AS_A - K_2GS_G$ (equation 2)

The following algebraic treatment allows the determination of the unknowns, K_1 and K_2. All symbols represent amounts per gram of mucosa.

Multiply equation 1 by S_G:

$dG/dt \text{ net } S_G = K_1AS_G - K_2GS_G$ and,

$K_2GS_G = K_1AS_G - dG/dt \text{ net } S_G$

Substituting the above value for K_2GS_G into equation 2:

3. $dG^*/dt \text{ net} = K_1AS_A - K_1AS_G + dG/dt \text{ net } S_G$ (equation 3)

Rewriting the term dG^*/dt net as follows:

$G^* \text{ net} = G \text{ net } S_G$

$dG^*/dt \text{ net} = d(G \text{ net } S_G)/dt$

$dG^*/dt \text{ net} = G \text{ net } dS_G/dt + S_G \, dG/dt \text{ net}$

Rewriting equation 3.

$G \text{ net } dS_G/dt + S_G \, dG/dt \text{ net} = K_1AS_A - K_1AS_G + dG/dt \text{ net } S_G$

and,

$G \text{ net } dS_G/dt = K_1AS_A - K_1AS_G$

and,

$$K_1 = \frac{G \text{ net}}{A} \frac{dS_G/dt}{(S_A - S_G)}$$ (equation 5)

Quantitative measurements of each parameter used in the above final equation (i.e., equation 5) were made on normal and bile deficient rats at the following postprandial time intervals: 1 hr., 2 hrs., 4 hrs., and 6 hours. Individual values for G/gm of mucosa, A/gm of mucosa, S_A, and S_G were used to calculate K_1 values for each rat; thus, it was necessary to use only the common value, dS_G/dt.

K_2 values were calculated by substitution of the previously calculated K_1 values into equation 1.

It should surely be mentioned that the equational analysis just described was not only based upon a conjectured physical 'system', but also upon the following assumptions:

1). That mucosal glyceride synthesis is a 'first order' reaction; that is, that all enzymes involved in the synthesis are functioning at sub-maximal capacity.

2). That the reaction fatty acid ——→ triglyceride is unidirectional; that is, that triglyceride is not hydrolyzed within the mucosal cell. It also assumes that triglyceride does not move from the lymph back into the cell.

3). That the availability of a-glycerophosphate is not a limiting factor.

B. General Procedure

1. Preparation of bile deficient rats.

Healthy Sprague-Dawley rats with body weights ranging from 257 to 407 grams were anesthetized with sodium pentobarbital (Nembutol; 50 mg/kg body weight) An abdominal incision was made which was large enough to permit access to the common bile duct and to the urinary bladder. The common bile duct was cannulated above its associations with the pancreatic duct using polyethylene tubing (PE 20). The cannula was made to lie along the dorsal side of the abdomen and the free end was inserted through the wall of the urinary bladder and stabilized there with a purse-string suture. The operated rats were placed in individual cages, given free access to food and water, and were allowed to recover for

five days before being used experimentally. The fecal material of all operated animals was tested for the presence of bile pigments one or two days before experimental use.[5] Animals which were not considered bile deficient or which had postoperative health problems were discarded.

2. Preparation of lipid 'meal' and feeding of rats.

All rats were fasted for 24 hours prior to experimental procedures. On the day of the experiment, all rats were fed 1 ml (by stomach tube under light ether anesthesia) of a mixture of 4 ml oleic acid labeled with tracer oleic acid-1-C^{14} and 2.857 gm fat-free milk powder. The labeled oleic acid used in the mixture was prepared in three 'batches'; the specific activity range was 181 dpm/mg to 201 dpm/mg. The specific activity of the oleic acid which was fed to each animal is listed in Tables 13 and 14, pages 64,65. The animals never had diarrhea nor did histological investigation show any signs of mucosal damage. The rats were killed at specified postprandial times by a blow on the head.

C. Removal of the Mucosa and Extraction of Mucosal Lipid.

Immediately after death the abdomen was opened and the intestinal lumen was flushed with 900 ml cold saline (4°C). All procedures involved with the rinsing of the intestine and the removal of mucosa were carried out in a cold room at 4°C. In anticipation of finding only small amounts of fatty acid within the mucosa, it was deemed absolutely essential to flush the intestinal lumen adequately; even small amounts of fatty acid adsorbed to the mucosa or carried in the mucous lining would be expected to introduce considerable error. The process of 'flushing' the intestinal

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5. The "Watson Qualitative Method for Detection of Urobilin and Urobilinogen in the Stool" was used. This method appears on pg. 86 of the Appendix.

lumen was as follows: 1. A large-bore hypodermic needle 2 cm in length was inserted into the duodenum at the junction of the duodenum with the pylorus of the stomach. A long rubber tube was attached to the external end of this needle so that the 'washings' (containing oleic acid-1-C^{14}) could be collected in a flask for disposal. 2. A second large-bore needle was inserted into the ileum just proximal to the junction of the ileum with the caecum. 3. The cold saline was injected into the intestinal lumen via syringe and the ileal needle at such a rate as to prevent undue distension of the intestine.

Following the flushing of the intestinal lumen, the intestine was ligated at each end and at 3 additional points at approximately quarterly intervals along the length of the intestine. After sectioning at the ligatures the segments were freed from the mesentery and were placed in a beaker of saline at 4° C. Working as quickly as possible, each segment was laid upon a cork board, split lengthwise, and pinned to the cork board with mucosa-side-up. Any obvious mucus remaining attached to the mucosa was gently wiped free with a damp gauze sponge. Finally, the mucosa was washed with an additional 25 ml portion of cold saline which was collected and analyzed for residual radioactivity. In all cases residual radioactivity was found to be negligible.

The mucosa was removed from the intestinal wall by gentle scraping with a spatula. As the mucosa collected upon the spatula it was placed in a stoppered and tared flask containing 30 ml methanol at 4° C. After the mucosa had been collected from all four segments of intestine, the tared flask containing methanol and mucosa was weighed and the weight of the mucosa calculated. Because of the chemistries involved, only

one experiment (i.e., one mucosal removal) was done at a time - except in cases where mucosal samples were pooled. These cases will be indicated in the section on Experimental Results. Mucosa was removed from the intestinal wall of 22 normal rats and 8 bile deficient rats.

The solvent system used for extraction of lipid from the mucosa was 2:1 chloroform:methanol[6]. Appropriate amounts of methanol and chloroform were added to the flask containing mucosa and methanol to bring the volume to 100 mls. The extraction was allowed to proceed over night. The following day the mixture was filtered and 90 mls (in a few instances, 80 mls) of the filtrate were placed in a separatory funnel. Five mls water for every 20 mls filtrate were added to the funnel and the mixture was well shaken and allowed to stand for 24 hours. After standing this length of time the lower chloroform layer had completely separated from the upper methanol-water layer and, hence, could be removed and saved. It was the chloroform layer which contained the extracted lipids. Following removal of the chloroform layer the remaining methanol-water mixture was twice 'washed' with added 2:1 chloroform:methanol, water was added, and the chloroform layer was again removed and added to that which had formerly been collected. After the chloroform layer had been collected from both 'washings' the chloroform was evaporated under nitrogen and the remaining lipid was ready to be fractionated into its components on silicic acid columns.

D. Preparation of Silicic Acid Columns and the Elution of Lipid

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6. The lipid extraction process used was a modification of the 'Sperry-Brand Method for Total lipid Extraction from Plasma.' Details of the method are given on page 87 of the Appendix.

Fractions.

1. A 6 gm silicic acid-KOH column was prepared as outlined on page 85 of the Appendix. The extracted lipid which remained after evaporation of chloroform was resuspended in ethyl ether (all ethyl ether used had been redistilled in glass) and placed on the column, as were several ethyl ether rinses of the flask. The solvent was allowed to flow through the column at the rate of 1.6 to 2.5 mls/minute. Neutral glycerides, cholesterol, and cholesterol ester were eluted with 175 mls ethyl ether. Free fatty acids were then eluted with 75 mls 3% formic acid in ethyl ether followed by 100 mls ethyl ether. Phospholipid was left upon the column. The elution system used was a slightly modified form of the system presented by McCarthy and Duthie (37).

2. A 10 gm silicic acid column was prepared (as outlined on page 86 of the Appendix) to separate the components of the first elution; i.e., to separate triglyceride from monoglyceride, cholesterol, and cholesterol ester. Positive pressures had to be applied to the column in order to maintain a solvent flow-rate through the column of approximately 2 mls/minute. After the ether solvent of the first elution had been evaporated the lipid which remained was resuspended in 3 mls cyclohexane and placed upon the column. The flask was washed 3 times with 3 ml portions of cyclohexane and these portions were also put upon the column. The cholesterol esters were eluted with 100 mls 3:2 cyclohexane:benzol and the triglyceride was eluted with 150 mls benzol. The solvent system used for elutions was suggested in a paper by Grimmer, et. al. (24). It is uncertain how well this elution system separates triglycerides from diglycerides.

E. Quantitative Analysis of Lipid Constituents.

1. The triglyceride and fatty acid eluants were evaporated to dryness, resuspended in ethyl ether, and transferred to 20 ml tared glass vials. The ethyl ether was evaporated and the recovery of triglyceride was quantitatively determined gravimetrically; the quantitative recovery of fatty acid was determined colorimetrically, the amount recovered being too small for precise gravimetric analysis.

The colorimetric determination of fatty acids utilized a micromethod as presented by Duncombe (17). Details of the method appear on page 88 of the Appendix. A standard curve was run with each set of unknowns. The method involves the formation of copper "soaps", followed by a colorimetric determination of copper.

2. Fifteen mls scintillation fluid[7] were placed in each glass vial and each vial was counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 314EX-2) for an interval of time sufficient to give a total count of 10,000. Efficiency of counting was calculated by recounting the samples after the addition of an 'internal standard' which carried a known number of disintegrations per minute. All radioactivity is expressed as net dpm, where net dpm = total dpm - background dpm.

F. Reliability and Validity of Methods.

1. Separation of fatty acid from all other lipids on a 6 gm silicic acid-KOH column.

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7. Recipe appears on page 90 of the Appendix.

Table 10.

Separate tests	lipid	Applied to Column total mgs	Applied to Column total dpm	Recovered from Column total mgs	Recovered from Column total dpm
1	Oleic acid-1-C^{14}	100.9	9164	99.5	9142
	Corn oil	102.7	0	104.3	87
2	Oleic acid-1-C^{14}	104.8	9679	112.5	9556
	triolein	50.0	0	136.3	0
	cholesterol	50.0	0		
	cholesterol palmitate	25.0	0		
	glyceryl monostearate	16.0	0		
	total	141.0			

Test 1, Table 10, indicated the following:

1. 98.6 per cent recovery of fatty acid by gravimetric analysis.
2. 99.8 per cent recovery of fatty acid by radioactive determination.
3. 101.6 per cent recovery of corn oil by gravimetric analysis.
4. The appearance of a small amount of radioactivity (87 dpm) in the recovered triglyceride indicated that < 1% of the oleic acid-1-C^{14} had been eluted in the triglyceride fraction.

Test 2, Table 10, indicated the following:

1. 107.3 per cent recovery of fatty acid by gravimetric analysis.
2. 98.7 per cent recovery of fatty acid by radioactive determination.
3. 96.7 per cent recovery of 'mixed' lipids by gravimetric analysis.

II. Efficiency of separation of triglyceride, monoglyceride, cholesterol, and cholesterol ester on a 10 gm silicic acid column.

Table 11

Separate tests	Lipid	Applied to Column total mgs	Applied to Column total dpm	Recovered from Column total mgs	Recovered from Column total dpm
1	Cholesteryl palmitate	19.8	0	20.5	32
	Tripalmitin-C^{14}	75.8	21,351	72.1	18,854
2	Tripalmitin	98.9	0	94.0	21
	Cholesterol-C^{14}	39.5	19,556	34.9	17,323
3	Tripalmitin-C^{14}	25.5	7,222	26.5	6,481
	Glyceryl monostearate	18.4	0	20.8	522

Test 1, Table 11, indicated the following:

1. 103.5 per cent recovery of cholesteryl palmitate by gravimetric analysis.
2. 95.1 per cent recovery of tripalmitin-C^{14} by gravimetric analysis.
3. 88.3 per cent recovery of tripalmitin-C^{14} by radioactive determination.
4. The appearance of a small amount of radioactivity (32 dpm) in the recovered cholesteryl palmitate indicated that 1% of the tripalmitin-C^{14} was eluted in the cholesteryl palmitate fraction.

Test 2, Table 11, indicated the following:

1. 95 per cent recovery of tripalmitin by gravimetric analysis.
2. 88.4 per cent recovery of cholesterol-C^{14} by gravimetric analysis.
3. 88.6 per cent recovery of cholesterol-C^{14} by radioactive determination.

4. The appearance of a small amount of radioactivity (21 dpm) in the recovered tripalmitin indicated that < 1% of the cholesterol-C^{14} was eluted with the tripalmitin.

Test 3, Table 11, indicated the following:

1. 103.9 per cent recovery of tripalmitin-C^{14} by gravimetric analysis.
2. 89.7 per cent recovery of tripalmitin-C^{14} by radioactive determination.
3. 113 per cent recovery of glyceryl monostearate by gravimetric analysis.
4. The presence of 522 dpm in the glyceryl monostearate fraction indicated that 1.84 mg labeled triglyceride had eluted in the glyceryl monostearate fraction; this is 7.2% of the amount of the labeled triglyceride added. In this experiment the lipids could not be 'washed' on to the column (with three 3 ml portions of cyclohexane), as was generally done, because of the almost complete insolubility of glyceryl monostearate in cyclohexane. This may have some relationship to the carry-over of tripalmitin-C^{14} into the glyceryl monostearate fraction.

III. Recoveries of known quantities of lipid which have been subjected to the complete experimental process; i.e., extraction and separation on silicic acid columns.

Table 12.

Separate tests	Lipid	Known Quantity of Lipid: total mgs	Known Quantity of Lipid: total dpm	Quantity of Lipid Recovered: total mgs	Quantity of Lipid Recovered: total dpm
1	Oleic acid-1-C^{14}	10.2	1745	9.195	1650
	Olive oil	19.95	0	19.0	89
2	Oleic acid-1-C^{14}	10.2	1745	9.917	1647
	Olive oil	19.95	0	18.867	91

Test 1, Table 12, indicated the following:

1. 90.1 per cent recovery of fatty acid by colorimetric analysis.
2. 94.5 per cent recovery of fatty acid by radioactive determination.
3. 95.2 per cent recovery of olive oil by gravimetric analysis.
4. The appearance of 89.4 dpm in the recovered olive oil indicated that 0.52 mg oleic acid-1-C^{14} eluted in the olive oil (triglyceride) fraction; this represents 5.1 per cent of the amount of oleic acid-1-C^{14} added.

Test 2, Table 12, indicated the following:

1. 97.2 per cent recovery of fatty acid by colorimetric analysis.
2. 94.4 per cent recovery of fatty acid by radioactive determination.
3. 94.6 per cent recovery of olive oil by gravimetric analysis.
4. As in Test 1, above.

It should be noted that, in experiments involving the analysis of lipids present in the mucosa, the range of total fatty acid recovered was 0.33 to 4.0 mg and the range of total triglyceride recovered was 1.2 to 24.4 mg.

RESULTS

As was discussed in the section on Methods and Materials, the accumulated data may be channeled in two directions: 1. The primary channel involves the use of the data in the equational analysis which was designed to allow measurement of the proportionality constant characteristic of mucosal triglyceride synthesis. This proportionality constant expresses milligrams of triglyceride synthesized per unit time in one gram of mucosa, per milligram of fatty acid present in one gram of mucosa, and is to be calculated for both normal and bile deficient rats. 2. If the proportionality constant values obtained from the equational analysis appear unreasonable, the secondary channel would involve the use of the data to re-evaluate the conjectures upon which the equational analysis was based.

A general discussion of the raw data will be presented in this section on Results, as will be the calculations from a primary use of the data, i.e., the proportionality constants evaluated from the proposed equational analysis. Two aspects of the proportionality constant evaluations indicated that the data should be used to re-evaluate the basis for the analysis. These two aspects will be presented at the end of the section on Results and the re-evaluation itself will be presented in the section on Discussion.

A. General discussion of the raw data.

The equational analysis required a measurement of the following six quantities. These six quantities were determined in rats which were sacrificed at postprandial time intervals of 1, 2, 4, and 6 hours.

1. Mgs fatty acid/gm mucosa.
2. Mgs triglyceride/gm mucosa.
3. Radioactivity (dpm's) present in 1.
4. Radioactivity (dpm's) present in 2.
5. Specific activity of mucosal fatty acid.
6. Specific activity of mucosal triglyceride.

The equation used to express the propcrtionality constant (K_1) of mucosal triglyceride synthesis appeared as $K_1 = \frac{G\ net}{A} \frac{dS_G/dt}{(S_A - S_G)}$. For this equation only the quantities represented above as 1., 2., 5., and 6. are directly pertinent. These quantities are outlined for normal and bile deficient rats in the Tables and Graphs presented in this section. It is recognized that the data on bile deficient rats offers a paucity of information which is utilizable in the equational analysis. As will be considered in the section on Discussion, the equational analysis is shown to inadequately represent the conditions of mucosal triglyceride synthesis; this inadequacy is revealed by a re-evaluation of the data from the normal animals. For this reason, and for the reason that utilization is not a criterion of validity, the data on bile deficient rats is considered below and along with the data presented on normal rats. The data presented were acquired from the analysis of mucosa from 22 normal rats (15 analyses; in some cases mucosal samples were pooled) and from 8 bile deficient rats.

For normal rats the ranges in mg fatty acid/gm mucosa at the indicated postprandial time intervals are notated in Table 13 (column 5) and are illustrated in Figure 7. Comparable values for bile deficient rats are notated in Table 14 (column 5) and are illustrated

in Figure 7. Values which are presented with an asterisk in Figure 7 indicate values obtained from rats which apparently absorbed negligible amounts of oleic acid-1-C^{14}, for insignificant amounts of radioactivity appeared in mucosal lipid. No explanation for the division of bile deficient rats into two different groups, i.e., those which absorbed the labeled fatty acid and those which did not, is offered. This division would seem a pertinent factor in the interpretation of experiments in which pooled mucosal samples were used; that is, values obtained in such an experiment would indicate a 'mean' which would be dependent upon the numbers of animals in the pool which had, or had not, absorbed the fed lipid. It should also be noted that, at the 4 hour postprandial interval, one bile deficient animal had inexplicably low mucosal fatty acid. It is tempting to postulate that this animal was not bile deficient, yet there is no experimental basis for the postulate.

It is apparent from Figure 7 that there was a significant difference in mgs fatty acid/gm mucosa between normal rats and those bile deficient rats which absorbed the oleic acid-1-C^{14}, at all postprandial time intervals.

It is interesting to note that in normal rats the amount of fatty acid/gm mucosa did not appear to increase significantly (slope = +0.028; coefficient of linearity = 0.39) through time. Turner, et. al., (56) fed a small amount of tripalmitin-C^{14} mixed in Purina Chow to normal rats; each meal contained 138 mg total lipid. They sacrificed 1 group of 12 rats at each of four postprandial intervals (1 hr., 3 hrs., and 8 hours), pooled the 12 mucosal samples at each interval, and extracted

and analyzed the pooled mucosal lipids. Their experiment is not comparable to the one reported in this Thesis because of differences in amount and type of lipid fed. It is of interest to point out, however, that their results also indicated a relatively constant mucosal fatty acid level. Data from their pooled samples were as follows:

Hours Postprandial	Total mg Fatty Acid
1	27.4
3	33.2
4	36.6
8	27.2

The authors suggested that the rather constant fatty acid level might function as one element in a feed-back system involved in the regulation of gastric emptying. If, for example, the mucosal fatty acid level increased markedly the suggested 'feed-back control' would modify (decrease) gastric emptying, with a subsequent decrease in the lipid available for absorption within the intestinal lumen. This postulated mechanism, however, does not appear to be adequate for the following reason: Two papers discussed in the Introduction (39,42) indicated that gastric emptying was more rapid in the absence of bile, yet the evidence presented here indicated that - in bile fistula rats - the mucosal fatty acid level was relatively high.

For normal rats the ranges in mg triglyceride/gm mucosa at the given postprandial time intervals are noted in Table 13 (column 6) and are illustrated in Figure 8. Comparable values for bile deficient rats are noted in Table 14 (column 6) and are illustrated in Figure 8. Those values associated with negligible absorption of oleic acid-1-C^{14}

again appear with an asterisk.

In both normal and bile deficient animals the change in triglyceride with time (dG/dt) is indicated by the slope of the curve passing through the approximate 'mean' triglyceride values at each time interval. The slope values (indicated by 'm' on Figure 8) are not used in the evaluation of 'K_1', yet it seemed of interest to include them. It is worth noting that the amount of triglyceride/gm mucosa in fasting bile deficient rats appeared to be significantly less than that present in fasting normal rats, as indicated by the amounts of triglyceride/gm mucosa present in rats which did not absorb oleic acid-1-C^{14} as well as by the slope (dG/dt) intercept with the ordinate. The changes in mg triglyceride/gm mucosa with time appeared to be quite comparable in normal and bile deficient rats.

The specific activities of the fatty acid extracted from normal mucosal samples are presented in Table 13 (column 7). The following two values were in evident error (apparently due to some analytical error which could not be traced): 1. The specific activity value noted with a symbol '+' was less than the specific activity of the triglyceride from the same mucosal sample; this is not possible since the fatty acid represents the precursor pool for triglyceride synthesis. 2. The specific activity value noted with the symbol '++' was higher than the specific activity of the fed oleic acid-1-C^{14}.

It is of interest to observe that in those rats involved in the postprandial intervals of 1 hr., 2 hrs., and 4 hrs. the specific activity of the mucosal fatty acids tends to remain (with the exception of 3 cases) considerably lower than the specific activity of the fed oleic

acid-1-C^{14}. This point will be considered again in the section on Discussion.

The specific activities of the fatty acid from bile deficient mucosal samples are presented in Table 14 (column 7). Again, two values were in evident error: 1. The specific activity value noted with the symbol '+' was less than the specific activity of the triglyceride extracted from the same mucosal sample. 2. The specific activity value noted with the symbol '++' was higher than the specific activity of the fed oleic acid-1-C^{14}.

The specific activities of the triglyceride extracted from normal mucosal samples are given in Table 13 (column 8) and are illustrated in Figure 9. The specific activities of the triglyceride extracted from bile deficient mucosal samples are given in Table 14 (column 8) and are illustrated in Figure 9. Linear regression lines have been established for both normal and bile deficient animals and are indicated on Figure 9. These regression lines indicate the value of the term dS_G/dt; it may be recalled that this value appears in the 'K_1' calculation. The linear regression line for normal animals had a slope of 5.907 and a coefficient of linearity of 0.659. The linear regression line for bile deficient animals had a slope of 13.61 and a coefficient of linearity of 0.9955.

B. Proportionality constants evaluated from the proposed equational analysis.

The 'K_1' and 'K_2' values calculated for individual normal and bile deficient rats are presented in Table 15; definitions of 'K_1' and 'K_2'

are included with the Table. The following considerations relevant to Table 15 are of interest.

1. The mean K_1 value for normal rats was 2.549 when the seemingly 'aberrant' individual K_1 value of 21.288 was excluded from the calculation of the mean. No explanation for the aberrant value is offered.
2. The mean K_1 value for bile deficient rats was 2.437 when the 'aberrant' individual K_1 value of 31.461 was excluded from the calculation of the mean. The aberrant value was associated with a rat with an abnormally low (relative to other bile deficient rats) mucosal fatty acid level.
3. The K_2 values for normal rats included negative values in the 1^{st} and 2^{nd} postprandial time intervals.

C. Aspects of the data which suggest a re-evaluation of the analysis.

Two aspects of the data in Table 15 suggested that the calculated 'K_1' values were erroneous. Such suggestions necessitated a re-evaluation of the premises upon which the equational analysis was based. The two aspects suggesting error were as follows: 1. Negative K_2 values, though they were small, are not compatible with the relationship dG/dt = triglyceride synthesized - triglyceride removed = $K_1A - K_2G$. 2. The mean K_1 values presented for normal and bile deficient rats were very similar (2.549 and 2.437, respectively). Since bile deficient rats exhibited a much higher mucosal fatty acid level than did normal rats, the consideration $dG/dt = K_1A$ would imply an increased triglyceride synthesis in bile deficient rats. As has been discussed

previously, investigators have never found indications of an increase in triglyceride synthesis in the absence of bile.

The utilization of the data in a re-evaluation of the premises upon which the equational analysis was based will be discussed in the section on Discussion.

Table 13

Data Relevant to Normal Rats

Hours post-prandial	Weight of rats (gms)	Specific Activity of fed oleic acid-C^{14} (dpm)	Weight of mucosa (gms)	Mucosal Lipids: mgs fatty acid per gm mucosa	Mucosal Lipids: mgs triglyceride per gm mucosa	Mucosal Lipids: Specific Activity of Fatty acids	Mucosal Lipids: Specific Activity of triglyceride
0*	420	0	1.040	.3076	2.989	0	0
	326	0	1.031	.5819	2.263	0	0
1	420,394,390**	184.0	4.772	.4388	5.108	82.744+	109.43
	290,312,294**	184.4	3.744	.6610	5.542	141.616	112.20
2	304.5	187.2	2.055	.5542	7.244	181.852	87.9
	387.0	185.2	1.8638	.3112	8.703	234.769++	121.42
	330.0	181.0	1.6547	.4986	8.461	142.909	94.3
	355, 361**	184.7	2.5462	.4277	6.546	128.111	115.93
4	371.5	183.2	1.1796	sample lost	9.797	sample lost	126.47
	371.0	181.3	1.2743	.6714	8.371	153.109	124.9
	320.0	181.6	1.1143	.4936	8.077	120.44	115.9
	349***	180.7	.944	.3840	8.3474	107.39	82.23
	304	187.6	.9985	.6927	11.6841	194.03	143.03
6	323,305**	186.0	2.2773	.7166	5.0498	183.358	131.80
	300,350**	185.6	2.1988	.4750	5.9123	178.463	131.688

* Fasting animals
** Mucosa from several animals was 'pooled'
*** Column trouble made data for this rat uncertain
\+ Specific Activity of fatty acid is lower than that of triglyceride
++ Specific Activity of Fatty acid is higher than that fed

Table 14

Data Relevant to Bile Deficient Rats

Hours post-prandial	Weight of rats (gms)	Specific Activity of fed oleic acid-C^{14} (dpm)	Weight of mucosa (gms)	Mucosal Lipids: mgs fatty acid per gm mucosa	Mucosal Lipids: mgs triglyceride per gm mucosa	Specific Activity of Fatty acids	Specific Activity of triglyceride
1	327	187.6	1.5186	1.0975	2.3413	53.78+	112.66
*	304	200.11	1.8129	.3125	1.1032	15.296	9.861
2	407	196.37	2.2178	1.7635	4.5089	136.17	119.79
*	277	194.76	1.6453	.4728	.7090	7.14	12.89
4	364	186.0	1.3890	.3759	9.000	170.51	160.15
	265	198.42	1.4527	2.4284	7.7250	157.786	145.228
6 *	257	201.7	1.4783	.3908	.9770	10.155	18.4248
	260	201.4	1.6927	1.1946	8.200	231.827++	178.000

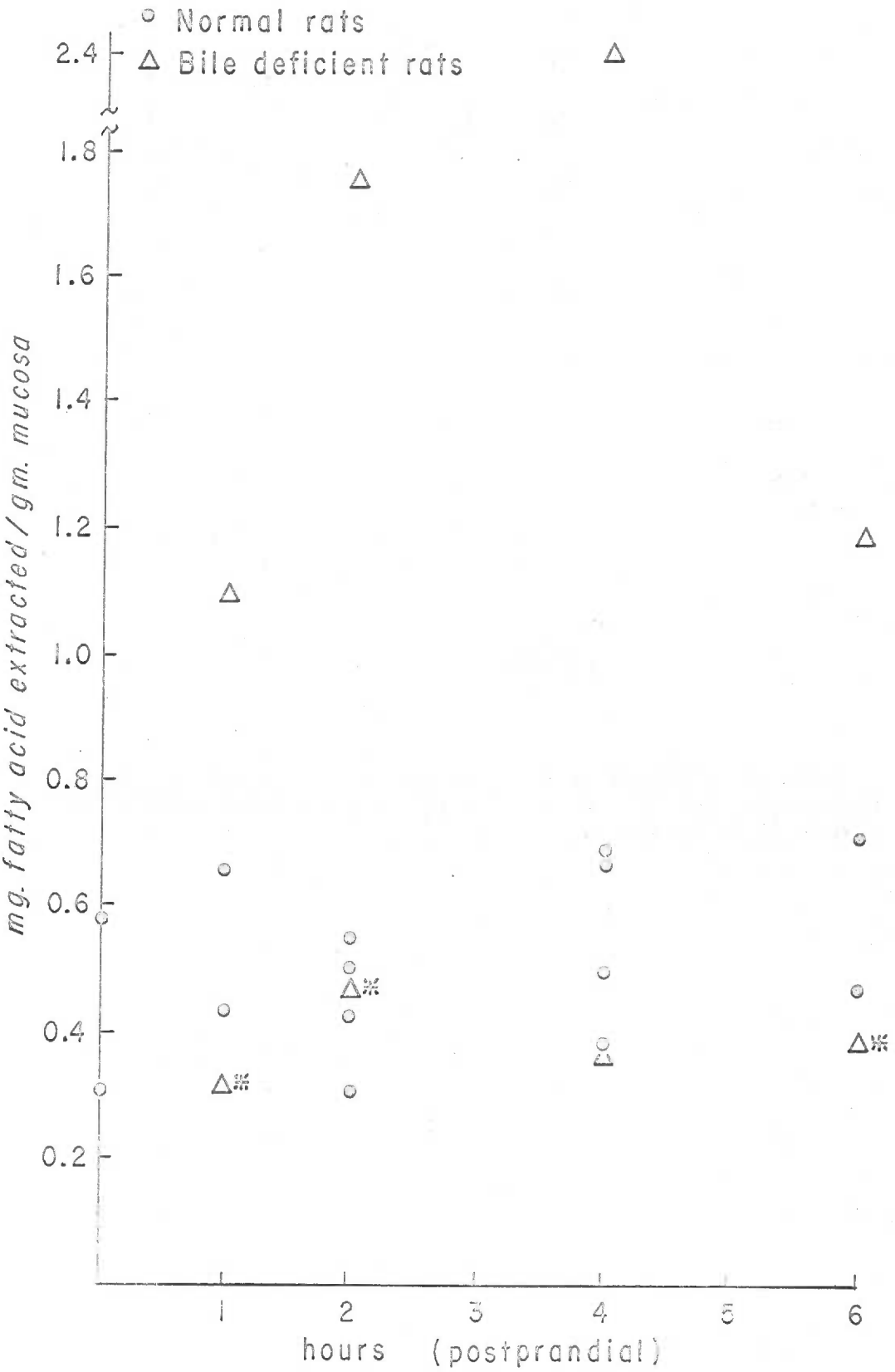
* Rats in which negligible radioactivity appeared in the mucosal lipids

\+ Specific activity of fatty acid is less than that of triglyceride

++ Specific activity of fatty acid is higher than that of the fed oleic acid-C^{14}

FIGURE 7

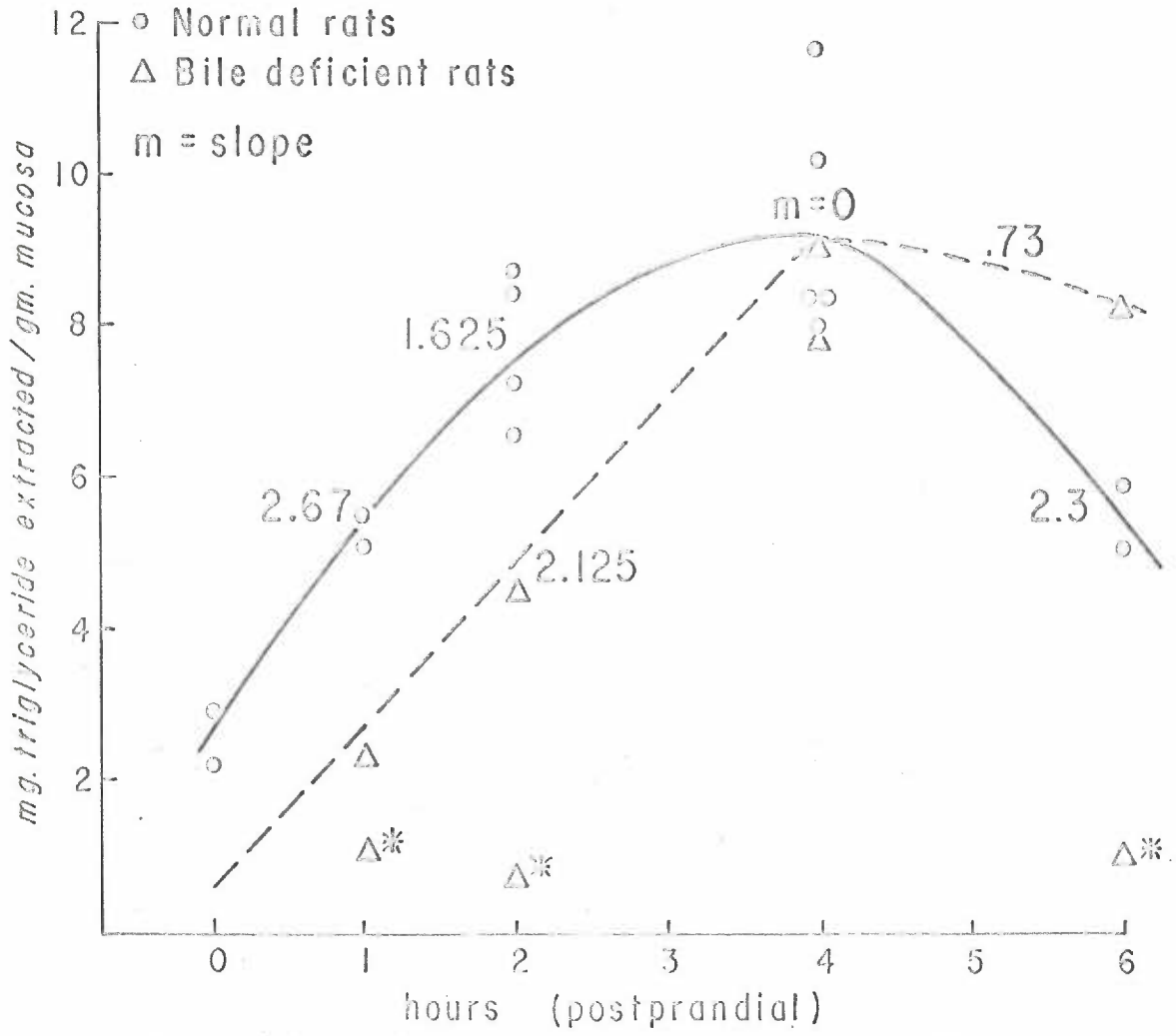
Mgs fatty acid extracted per gram of mucosa are placed for individual normal and bile deficient rats at appropriate post-prandial time intervals.



* Negligible radioactivity appeared in mucosal lipids

FIGURE 8

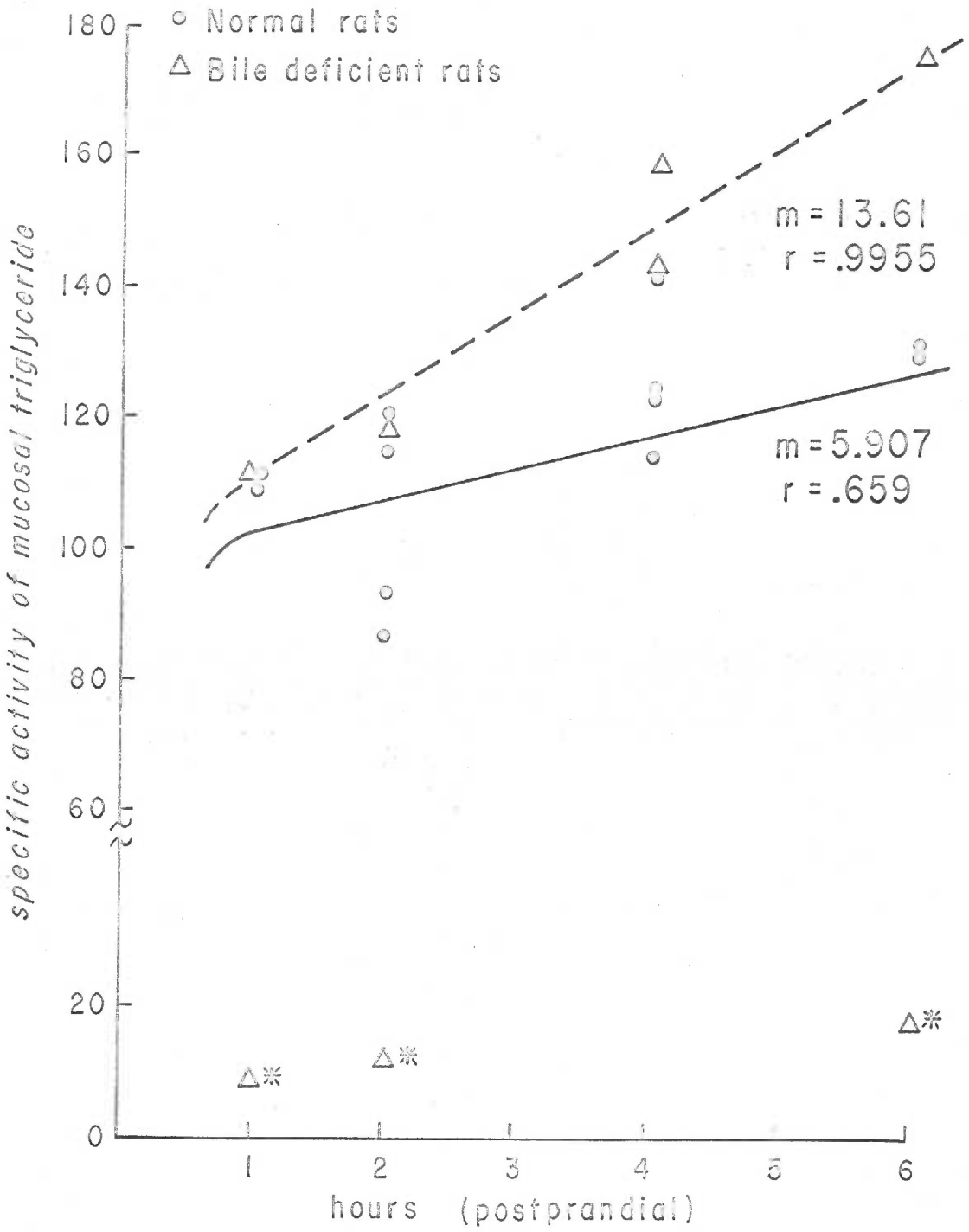
Mgs triglyceride extracted per gram of mucosa are graphed for individual normal and bile deficient rats at appropriate postprandial intervals. The change in mgs triglyceride with time (dG/dt) is indicated by the slope of the curve passing through the approximate 'mean' triglyceride values at each time interval; the slope values are indicated with the symbol 'm'.



* Negligible radioactivity appeared in mucosal lipids

FIGURE 9

Specific activities of the triglyceride extracted from mucosal samples taken from normal and bile deficient rats sacrificed at the indicated postprandial times.



m = slope

r = coefficient of correlation (a measure of degree of linear relationship between 2 variables)

* Negligible radioactivity appeared in mucosal lipids

Table 15.

K_1* and K_2** Values For Normal and Bile Deficient Rats.

I. Normal Rats

Hours post-prandial	K_1 values for individual rats	K_2 values for individual rats
1	1.677	-0.289
2	0.823	-0.161
	1.454	-0.134
	2.062	-0.070
	7.423	0.236
4	2.606	0.209
	21.288	1.301
	5.102	0.234
	1.957	0.115
6	0.8125	0.570
	1.568	0.515

II. Bile Deficient Rats

Hours post-prandial	K_1 values for individual rats	K_2 values for individual rats
2	2.125	0.359
4+	31.461	1.313
	3.448	1.083
6	1.737	0.341

* K_1 defined as = $\frac{\text{mgs triglyceride synthesized per unit time/gm mucosa}}{\text{mgs fatty acid/gm mucosa}}$

** K_2 defined as = $\frac{\text{mgs triglyceride removed per unit time/gm mucosa}}{\text{mgs triglyceride/gm mucosa}}$

\+ Case associated with abnormal mucosal fatty acid level

DISCUSSION

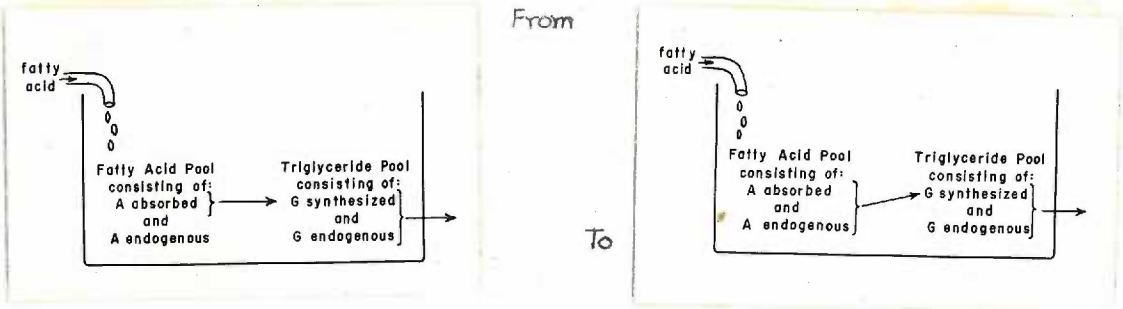
The section on Results channeled the accumulated data into calculations of the proportionality constants characterizing mucosal triglyceride synthesis in both normal and bile deficient rats. Two aspects of these calculations suggested that the data be channeled into a re-evaluation of the premises upon which the equational analysis was based. Such a channel comprises the secondary use of the data and is not without value for, as was previously discussed (page 42), such a re-evaluation provides information upon aspects of lipid absorption which have been neglected. For example, information becomes available upon changes occurring in endogenous pool sizes during active fatty acid absorption and mucosal triglyceride synthesis.

Changes in the proposed 'system' upon which the equational analysis was based will be discussed individually.

1. The total dpm of individual mucosal fatty acid fractions, when divided by the specific activity of the fed fatty acid, indicated the amount of absorbed fatty acid which was present in the mucosa. Subtraction of the amount of absorbed fatty acid from the total amount of fatty acid present, yielded a value which represented the size of the unlabeled mucosal fatty acid pool. When these calculations were made for individual fed normal rats, it was observed that the amount of unlabeled fatty acid present (per gram of mucosa) frequently appeared to be about one-fourth of the amount found in normal fasting animals (see Table 17, page 91 of the Appendix). Since this value remained quite consistent

over the one, two, and four hour postprandial periods, it appeared to represent 'inactive' fatty acid which might, perhaps, be associated as a lipo-protein structural component of the mucosa. The originally conjectured physical 'system' (at the postprandial time intervals at which it was observed) might, then, be altered as follows:

Figure 10.



The equation for triglyceride synthesis would, then, become:

dG/dt synthesized $= K_1A'$, where A' represents the size of the 'active' fatty acid pool.

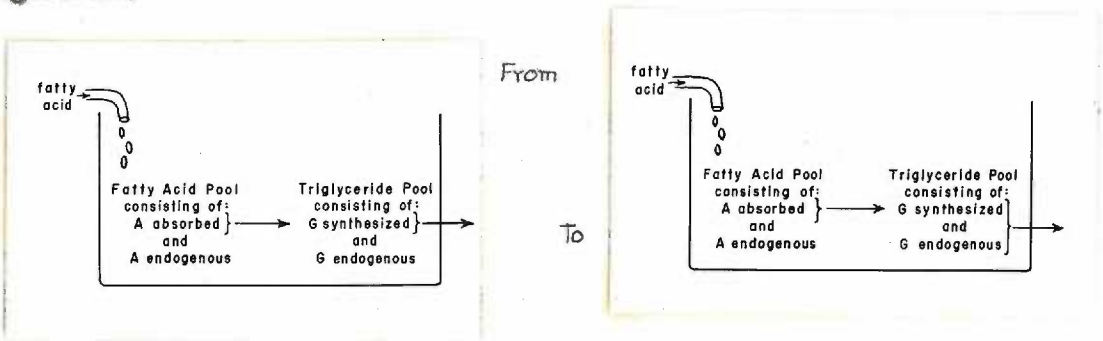
Since, for the one, two, and four hour postprandial periods, the amount of unlabeled fatty acid appeared to remain about the same and, since the specific activity of mucosal fatty acid did not appear to be increasing (with some exceptions) through the postprandial time span of 1 - 4 hours, the 'active' fatty acid pool might be considered to consist of absorbed fatty acid alone and, hence, to be represented by the specific activity of the fed fatty acid. Then,

dG^*/dt synthesized $= K_1A'S_A$, where S_A equals the specific activity of the fatty acid fed.

2. The total dpm of individual triglyceride fractions, when divided

by the specific activity of the fed fatty acid, indicated the mgs of the fed fatty acid which had been synthesized into triglyceride. When these amounts (for individual normal rats) were plotted against the total mgs of triglyceride present (see Figure 12, page 92 of the Appendix), it appeared that the amount of unlabeled triglyceride did not change greatly over the 1 - 4 hour postprandial periods. In this case also, it might be suggested that this indicated that the unlabeled triglyceride represented an 'inactive' triglyceride pool which could be associated with structural glyceride or proteolipid. If the unlabeled triglyceride pool remains of constant size, the triglyceride removed must be composed of synthesized triglyceride. Again, it would seem that the original proposal for the 'system' involved in mucosal triglyceride synthesis should be altered as follows:

Figure 11.



Triglyceride removal, then, might best be considered as some function of the amount of triglyceride synthesized, or,

$$dG/dt \text{ removed} = K_2 (K_1 A')$$

Since the triglyceride synthesized must have the same specific activity as its presursor ('active' fatty acid) pool and, since the triglyceride removed is composed of synthesized triglyceride,

then:

$$dG^*/dt \text{ removed} = K_2 (K_1A') S_G = K_2 (K_1A') S_A$$

This represents a seemingly insoluble problem, for the final equational analysis becomes:

1. $dG/dt \text{ net} = K_1A' - K_2 (K_1A')$
2. $dG^*/dt \text{ net} = K_1A'S_A - K_2 (K_1A') S_A$

The above pair of equations do not incorporate enough parameters to allow their solution simultaneously. Let us, then consider the experimental data in somewhat general terms.

Several interesting observations are possible with regard to the 'tracing' of endogenous lipid pool sizes during fatty acid absorption and mucosal triglyceride synthesis: 1. The amount of endogenous fatty acid present in the mucosa of oleic acid fed normal rats was not the same as that found in fasted normal rats. Previous investigators (33) have made the assumption that amounts of endogenous lipid do not change during the absorptive processes. 2. Endogenous amounts of fatty acid found in 5 of the 8 oleic acid fed bile fistula rats did not appear to change beyond the normal range of amounts found in normal fasted rats. 3. There was some indication that, though amounts of endogenous triglyceride did not change in oleic acid fed normal rats through the 1 - 4 postprandial periods, a decrease may have occurred at the 6 hour period. These observations indicate that experiments designed to determine the significance of the changes in endogenous pool sizes occurring over a long postprandial time interval would be of value.

Before closing the discussion of experimental data, it might be of interest to compare the data from the normal rats with that from

the bile deficient rats in a manner similar to that presented in a paper by Knoebel and Ryan (33). It may be recalled that this paper was discussed in the Introduction and Review of the Literature (pages 35-37). These authors fed normal and bile deficient dogs a meal which contained unlabeled oleic acid. At 4 hours postprandial they sacrificed the dogs, removed the mucosa, extracted the mucosal lipid, and fractionated the extracted lipid into its components. They observed that, in bile deficient dogs, the fatty acid represented a greater percentage of the total mucosal lipid than was the case in normal dogs. From this observation the authors concluded that their data indicated impaired mucosal triglyceride synthesis in bile deficient dogs. The actual percentages were as follows:

	Bile Deficient % fatty acid	Normal % fatty acid
Duodenal-jejunal mucosa	27.4	19.6
Ileal mucosa	19.7	11.5

Treatment of the data presented in this Thesis in a manner similar to the presentation by Knoebel and Ryan leads to Table 16. It will be noted that column 2 represents the summed weight of the labeled fatty acid and labeled triglyceride extracted per gram of mucosa. In column 5 are listed (for individual animals) the values expressing labeled fatty acid as a percentage of the summed labeled fatty acid and triglyceride. The mean percentage for normal animals was 8.5; the mean percentage for bile deficient animals was 22.5. Consideration of the data in these terms leads to a conclusion comparable to that of Knoebel and Ryan; i.e., an impairment of mucosal triglyceride synthesis was

indicated in the bile deficient animal.

Table 16.
Weight of Labeled Fatty Acid Expressed as a Percentage of the Summed Weight of Labeled Fatty Acid and Labeled Triglyceride, For Individual Normal and Bile Deficient Rats.

Normal Rats

Hours post-prandial	Summed weight of labeled fatty acid and labeled triglyceride	Weight of labeled fatty acids	Weight of labeled triglyceride	Labeled fatty acid as % of 'summed' (col. 2) labeled lipid
	mg/gm of mucosa	mg/gm of mucosa	mg/gm of mucosa	%
1	4.29	0.51	3.78	11.9
2	4.32	0.54	3.78	12.5
	6.73	0.395	6.33	5.9
	5.29	0.394	4.89	7.5
	4.86	0.302	4.56	6.2
4	7.01	0.567	6.44	8.1
	6.0	0.327	5.67	5.5
	4.45	0.288	4.22	5.1
	10.61	0.716	9.89	6.8
6	4.71	0.706	4.0	15.0
	5.13	0.457	4.67	8.9
Bile Deficient Rats				
1	1.876	0.314	1.56	16.3
2	3.79	1.22	2.75	32.2
4	7.58	1.93	5.65	25.5
6	8.62	1.38	7.24	16.0

SUMMARY

Several parameters reflecting events occurring during intestinal mucosal synthesis of triglyceride from absorbed fatty acid have been studied in normal and bile deficient rats. Rats in the bile deficient group were deprived of bile by surgical implantation of cannulae leading from bile duct to urinary bladder. Rats exposed to surgery were not used experimentally until the fifth postoperative day. Individual normal and bile deficient rats were fed 575 mgs oleic acid containing tracer oleic acid-1-C^{14}. At postprandial periods of from 1 to 6 hours the animals were sacrificed and the mucosa was gently scraped from the intestinal wall. Lipids contained within the mucosa were extracted and quantitative separation of the fatty acid and triglyceride fractions was obtained with silicic acid columns. The following quantitative measurements (for individual rats) were made upon the fractionated mucosal lipids: Mgs fatty acid/gm of mucosa, mgs triglyceride/gm of mucosa, specific activity of the fatty acid, and specific activity of the triglyceride. Mucosa was removed from the intestinal wall of 22 normal rats (15 analyses; in some cases mucosal samples were pooled) and from 8 bile deficient rats.

It was proposed that the above quantitative measurements be used in the following two capacities: 1. A capacity designed for use of the measurements in an equational analysis intended to provide a solution to the current question, "do bile salts have an intracellular function?". The equational analysis was based upon a use of the law of mass action and involved the calculation of the proportionality

constant characterizing the mucosal triglyceride synthesis in individual rats The proportionality constant was symbolized as K_1 and was defined as follows: $K_1 = \frac{\text{mgs triglyceride synthesized/unit time}}{\text{mg fatty acid}}$,

where each quantity used in the calculation represented an amount per gram of mucosa. It was proposed that a comparison of K_1 values obtained for normal rats with those obtained for bile deficient rats would allow a definitive solution to the questioned role of bile in mucosal triglyceride synthesis. 2. Since the literature affords little, if any, information on the kinetics of in vivo mucosal triglyceride synthesis, the equational analysis forming the basis for the above calculations was dependent upon several assumptions and conjectures. It was proposed that, if the calculations indicated errors in the equational analysis, the data could be used in a second capacity, i.e., in the possible identification of deficiencies in the assumptions and/or conjectures. Although the use of the data in this capacity would not resolve the question of bile action, it would contribute to information relevant to the intracellular conditions prevailing during active fatty acid absorption and synthesis of triglyceride.

The results of this investigation were as follows:

1. In normal animals the average mucosal fatty acid increased only slightly with time, while net mucosal triglyceride increased prior to the 4th postprandial hour and decreased from the 4th through the 6th postprandial hour.
2. Data from the bile deficient animals were limited by the absence of fatty acid absorption in three cases. In four of the five

animals which absorbed the fed oleic acid the amount of fatty acid found in the mucosa was significantly higher than that found in normal animals. The net mucosal triglyceride in the five animals which absorbed the fed oleic acid increased prior to the 4^{th} postprandial hour and decreased from the 4^{th} through the 6^{th} postprandial hour, though the increase was linear with time in contrast to an apparent non linear increase in normal animals.

3. The increase in the specific activity of mucosal triglyceride was linear in both normal and bile deficient animals after the 1^{st} postprandial hour. The slope of the specific activity of mucosal triglyceride vs. time was 5.907 for normal animals and 13.61 for bile deficient animals. The coefficient of correlation for each curve was 0.659 and 0.9955, respectively.

4. The following two aspects of K_1 calculations suggested that the 'system' proposed to be involved in mucosal triglyceride synthesis be re-evaluated in the light of the collected data: 1. Since it was found that the proportionality constant for mucosal triglyceride synthesis in bile deficient rats (in those which absorbed oleic acid) was comparable to that found for normal rats, and since the mucosal amount of precursor (fatty acid) found in these bile deficient rats was much higher than that found in normal mucosa, it would be expected that bile deficient animals would exhibit an increased quantitative triglyceride synthesis. Prior investigations have never indicated that this might occur. 2. Values for a proportionality constant expressing the removal

of triglyceride from the mucosal cell were found to be negative for normal rats at the 1 and 2 hour postprandial time intervals. Such negative values were not compatible with the proposed equational analysis.

5. Re-evaluation of the proposed conjectured system and the equational analysis based upon it in the light of the accumulated data indicated that unforseen changes in endogenous fatty acid and triglyceride pools prevented a use of the equations in their proposed form. Re-evaluation of the data for normal rats indicated that in six of the nine analyses made during the 1, 2, and 4 postprandial intervals the endogenous fatty acid had decreased to a value of approximately ½ of the amount found in fasting mucosa. In all normal rats the endogenous mucosal triglyceride did not change from fasting levels throughout the 1 - 4 hour postprandial interval.

6. It may be concluded that there are demonstrable differences in the measured variables between normal and bile deficient rats and that, though the equational analyses designed to quantitate this difference did not prove feasible, the analysis was considered to be justifiable and informative.

BIBLIOGRAPHY

1. Agar, W. T., Bird, F. J. R., and Sidhu, G. S. The uptake of amino acids by the intestine. Biochim. Biophys. Acta, 1954. 14, 80-84

2. Ashworth, C. T., and Johnston, J. M. The intestinal absorption of fatty acid: A biochemical and electron microscope study. J. Lipid Research, 1963. 4, 454-60

3. Baker, R. D., and Searle, G. W. Bile salt absorption at various levels of rat small intestine. Proc. Soc. Exp. Bio. and Med., 1960. 105, 521-23

4. Bergström, S., Blomstrand, R., and Borgström, B. Route of absorption and distribution of oleic acid and triolein in the rat. Biochem. J., 1954. 58, 600-604

5. Bergström, S., and Borgström, B. Metabolism of lipids. Annual Review of Biochemistry, 1956. 25, pg 191

6. Bergström, S., Borgström, B., and Carlsten, A. On the mechanism of intestinal fat absorption in the cat. Acta Physiol. Scand., 1954. 32, 94-98

7. Bergström, S., Borgström, B., Carlsten, A., and Rottenberg, M. On the mechanism of intestinal fat absorption. Acta Chemica Scand., 1950. 4, 1142-43

8. Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G. The quantitative significance of the lymphatic pathway in transport of absorbed fatty acids. J. of Biol. Chem., 1950. 184, 1-8

9. Borgström, B. On the mechanism of the intestinal fat absorption. IV. Metabolism of lipids. Acta Physiol. Scand., 1952. 25, 291-314

10. Borgström, B. On the mechanism of the intestinal fat absorption V. The effect of bile diversion on fat absorption in the rat. Acta Physiol. Scand., 1953. 28, 279-86

11. Borgström, B. Effect of tauro-cholic acid on the pH/activity curve of pancreatic lipase. Biochim. Biophys. Acta, 1954. 13, 149-50.

12. Borgström, B. Studies on pancreatic lipase. In G. Popjak and E. Le Breton (ed.) Biochemical Problems of Lipids. New York: Interscience Publishers, Inc., London: Butterworths Scientific Publications, 1956. pp. 179-86

13. Clark, B., and Hübscher, G. Biosynthesis of glycerides in subcellular fractions of intestinal mucosa. Biochim. Biophys. Acta, 1961. 46, 479-94

14. Danielson, H. Influence of bile acids on digestion and absorption of lipids. Am. J. Clin. Nutrition, 1963. 12, 214-18

15. Dawson, A. M., and Isselbacher, K. J. The esterification of palmitate-1-C^{14} by homogenates of intestinal mucosa. J. of Clin. Investigation, 1960. 39, 150-60

16. Dawson, A. M., and Isselbacher, K. J. Studies on lipid metabolism in the small intestine with observations on the role of bile salts. J. of Clin. Investigation, 1960. 39, 730-40

17. Duncombe, W. G. The colorimetric micro-determination of long-chain fatty acids. Biochem. J., 1963. 88, 7-10

18. Forbes, O. C., and Lines, J. C. Some effects of bile shunting in rats. In A. C. Frazer (Ed.) Biochem. Problems of Lipids. Amsterdam, London, and New York: Elseveir Publishing Co., 1963. pp. 60-65

19. Frazer, A. C. Fat absorption and its relationship to fat metabolism. J. Physiol., 1940. 20, 561-81

20. Frazer, A. C. Differentiation in the absorption of olive oil and oleic acid in the rat. J. Physiol., 1943. 102, 306-12

21. Frazer, A. C. The absorption of triglyceride fat from the intestine. Physiol. Rev., 1946. 26, 103-19

22. Frazer, A. C., and Sammons, H. G. The formation of mono- and diglycerides during the hydrolysis of triglyceride by pancreatic lipase. Biochem. J., 1945. 89, 122-28

23. Frazer, A. C., Schulman, J. H., and Stewart, H. C. Emulsification of fat in the intestine of the rat and its relationship to absorption. J. Physiol., 1944. 103, 306-16

24. Grimmer, G., Glaser, A., Oertel, H., Boigt, K., and Apostolakis, M. Vergleich der Fettsaurezusammensetzung der Cholesterinester, Triglyceride, Phosphatide und freien Fettsauren in Blut und Lymphe von Ratten. Hoppe-Seylers Zeitschrift f. Physiol. Chemie, 1963. 333, 232-41

25. Haslewood, G. A. D. Recent developments in our knowledge of bile salts. Physiol. Revl, 1955. 35, 178

26. Heersma, J. R., and Annegers, J. H. Effect of bile diversion on fecal fat and nitrogen excretion. Am J. Physiol., 1946. 153, 143-47

27. Heerema, J. R., and Annegers, J. H. Effect of bile preparations on fat absorption in bile fistula dogs. Proc. Soc. Exp. Biol. and Med., 1948. 67, 339-40

28. Hofmann, A. F., and Borgström, B. Physico-chemical state of lipids in intestinal content during their digestion and absorption. Fed. Proc., 1962. 21, 43-50

29. Isselbacher, K. J., and Senior, J. R. The intestinal absorption of carbohydrate and fat. Gastroenterology, 1964. 46, 287-98

30. Johnston, J. M. Site of fatty acid absorption. Proc. Soc. Exp. Biol. and Med., 1959. 100, 669-70

31. Johnston, J. M., and Borgström, B. The intestinal absorption and metabolism of micellar solutions of lipids. Biochim. Biophys. Acta, 1965. 84, 412-23

32. Keeney, M. A survey of United States butterfat constants. II. Butyric acid. J. Assoc. of Official Agricultural Chemists, 1956. 39, 212-25

33. Knoebel, L. K., and Ryan, J. M. Digestion and mucosal absorption of fat in normal and bile deficient dogs. Am. J. Physiol., 1963. 204, 509-14

34. Kornberg, A., and Pricer, W. E., Jr. Enzymatic synthesis of phosphorus-containing lipids. J. Amer. Chem. Soc., 1952. 74, 1617

35. Kornberg, A., and Pricer, W. E., Jr. Enzymatic synthesis of the co-enzyme A derivatives of long-chain fatty acids. J. Biol. Chem., 1953. 204, 329-43

36. Lacy, D., and Taylor, A. B. Fat absorption by epithelial cells of the small intestine of the rat. Am. J. Anatomy, 1962. 110, 155-63

37. McCarthy, R. D., and Duthie, A. H. A rapid quantitative method for the separation of free fatty acids from other lipids. J. lipid Research, 1962. 3, 117-19

38. Mellanby, J. The digestion and absorption of fat. J. Physiol., 1927. 64, Proc. v.

39. Menguy, R. Role of biliary and pancreatic secretions in the inhibition of gastric motility by fat in the intestine. An experimental study in the rat. Am J. Dig. Diseases, 1960. 5, 792-800

40. Morgan, R. G. H. The effect of diverting bile and pancreatic juice on the inhibition of gastric motility by duodenal stimuli in the unanesthetized rat. Quart. J. Exp. Physiol., 1963. 48, 273-77

41. Morgan, R. G. H. The effect of bile salts on the lymphatic absorption by the unanesthetized rat of intraduodenally infused lipids. Quart. J. Exp. Physiol., 1964. 49, 1957-65

42. Morgan, G. H., and Simmonds, W. J. The relative effects of diversion of bile to the ileum or to the urinary bladder on fat absorption and gastrointestinal motility in the rat. Quart. J. Exp. Physiol., 1962. 47, 352-59

43. Palay, S. L., and Karlin, L. J. An electron microscopic study of the intestinal villus. II. The pathway of fat absorption. J. Biophys. and Biochem. Cytology, 1959. 5, 373-84

44. Pearse, A. G. E. Histochemistry, theoretical and applied. Second edition. Boston: Little, Brown, 1960

45. Pessoa, V. C., Kim, K. S., and Ivy, A. C. Fat absorption in the absence of bile and pancreatic juice. Am J. Physiol., 1953. 174, 209-18

46. Pflüger, E. Fortgesetzte Untersuchnung uber die in wasserloslicher Form sich vollziehende Resorption der Fette. Pfluger, Archiv. fur Physiologie, 1902. 88, 299-338

47. Redman, T., Willimott, S. G., and Wokes, F. The pH of the gastrointestinal tract of certain rodents used in feeding experiments, and its possible significance in rickets. J. Biochem., 1927. 21, 589-605

48. Reiser, R., Bryson, M. J., Carr, M. J., and Kuiken, K. A. The intestinal absorption of triglycerides. J. Biol. Chem., 1952 194, 131-38

49. Riegel, C., O'Shea Kisom, K., and Ravdin, I. S. The influence of sodium taurocholate, hepatic bile and gall-bladder bile upon the absorption of oleic acid from the small intestine. Am J. Physiol., 1935. 112, 669-72

50. Saunders, D. A., and Dawson, A. M. The absorption of oleic acid in the bile fistula rat. Gut, 1963. 4, 254-60

51. Saunders, D. A., and Dawson, A. M. The effect of bile diversion on the absorption of U-C^{14} oleic acid in the rat. In A. C. Frazer (Ed.) Biochemical Problems of Lipids. Amsterdam, London, New York: Elsevier Publishing Co., 1963. pp. 55-59

52. Senior, J. R. Intestinal absorption of fats. J. Lipid Research, 1964. 5, 495-521

53. Smith, S. W., Weiss, S. B., and Kennedy, E. P. The enzymatic dephosphorylation of phosphatidic acids. J. Biol. Chem., 1957. 228, 915-922

54. Spencer, R. P. Spatial distribution of intestinal activities. Yale J. Biol. and Med., 1963-64. 36, 279-94

55. Sperry, W. M., and Brand, F. C. The determination of total lipides in blood serum. J. Biol. Chem., 1955. 213, 69-76

56. Turner, D. A., Cox, E. V., Balint, J. A., Pirrie, R., Fletcher, R. F., Huang, E., and Cevallos, W. H. Digestion and absorption of fat after a normal meal. Fed. Proc., 1960. 19, 876-83

57. Wilson, T. H., and Wiseman, G. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol., 1954. 123, 116-25

58. Verzár, F. Absorption from the intestine. Monographs on Physiology. London, Toronto, and New York: Longmans, Green and Co., 1936

APPENDIX

A. Preparation of a 6 gm silicic acid-KOH column.

1. Isopropanol-KOH reagent was prepared in the following way (32): 25 gms KOH (85%) pellets were dissolved in 400 mls isopropanol by warming and swirling on a steam bath. The supernatant isopropanol-KOH solution was decanted from the small amount of aqueous KOH adherent to the bottom of the flask. The decanted isopropanol-KOH solution (containing approximately 50 mg KOH/ml) was cooled and stored in the refrigerator.
2. Following the method of McCarthy and Duthie (37), Mallinckrodt silicic acid, 100 mesh, was 'washed' to remove the coarser particles; this allowed improved flow through the column. The silicic acid was washed by suspending 100 gms silicic acid in 400 mls methanol and decanting off any particles which did not settle in 5 minutes. This was repeated once with methanol and once with 400 mls acetone. Finally, the silicic acid was rinsed with redistilled ethyl ether, permitted to dry in air, and stored in a desicator.
3. Six gms of prepared silicic acid were weighed into a small beaker. To this was added, with mixing, 12 mls isopropanol-KOH reagent and 36 mls redistilled ethyl ether. After standing 5 minutes, the silicic acid slurry was poured into a glass chromatographic column (16 mm in diameter, 25 cm high) and washed with 100 mls redistilled ethyl ether. A new column was prepared for each experiment. Cold tap water was circulated

at all times through the column jacket when the column was in use.

B. Preparation of a 10 gm silicic acid column.

1. Silicic acid (400 mesh; Bio-Rad Laboratories) was 'activated' by heating in a 115° C. oven overnight. For preparation of a column, 10 gms 'activated' silicic acid were slurried in approximately 30 mls cyclohexane. The slurry was poured into a glass chromatographic column (16 mm in diameter, 25 cm high) and allowed to settle with no application of positive pressure. The walls of the column were washed free of adherent silicic acid with small portions of cyclohexane. A new column was prepared for each experiment. Cold tap water was always circulated through the jacket of the column when the column was in use.

C. A modified 'Watson Qualitative Method for Detection of Urobilin and Urobilinogen in the stool'.

Urobilin and urobilinogen were extracted from the stool with alcoholic acetate. The amount of green fluorescence in the extract may be used as an index of the amount of urobilin present in the specimen. Any stool may be used for the urobilin test, but fresh stool is required for the urobilinogen test. Only the presence of urobilinogen was tested for in this paper.

1. Method: About 2 gms fresh stool were emulsified with 10 mls alcoholic Zinc acetate. The emulsion was filtered and 2.5 mls of the filtrate were mixed with 2.5 mls Ehrlich's aldehyde reagent. After 15 seconds, 5 mls of saturated aqueous Sodium

acetate were added. A slowly developing pink color was indicative of the presence of urobilinogen.

2. Reagents:

a) Zinc acetate: 50 gms Zinc acetate were added to 1 liter of 95% ethyl alcohol. The solution was heated to 50°C. and allowed to cool before use.

b) Ehrlich's aldehyde reagent: 0.7 gm p-dimethylaminobenzaldehyde was dissolved in 150 mls concentrated HCl. 100 mls distilled water were added. Reagent was stable for several months if stored in a brown bottle.

c) Sodium acetate(saturated solution): Sodium acetate was added to water and the solution heated to 60°C. When cool the solution contained a large excess of crystals.

D. Chloroform:Methanol extraction (lipid) procedure; modified form of Sperry and Brand 'Plasma Total Lipid Extraction' method. (55)

1. An amount of methanol appropriate to a final volume of chloroform:methanol 2:1 was placed in a volumetric flask. The lipid-bearing material (mucosa) was added to the methanol, as were a few mls chloroform, and the whole was brought to a boil on a water bath. The lipid-bearing material was extracted with approximately 25 times its weight of extraction fluid. After removal from the water bath, the samples were brought almost to volume with chloroform, 3 drops of 0.1 N HCl/25 mls total volume were added (to convert the soaps to fatty acids), and the mixture was allowed to stand for at least 12 hours at room temperature. After standing the mixture was brought to volume

with chloroform, shaken, and filtered as rapidly as possible (to avoid evaporation of solvent) through No. 2 Whatman filter paper. Ninety mls (in some cases, 80 mls) of filtrate were placed in a separatory funnel. Five mls water/20 mls filtrate were added and the funnel was stoppered and shaken vigorously. Separation of the chloroform layer from the methanol-water layer was accomplished by allowing the funnel to stand for 24 hours. The chloroform layer was subnatant in the separatory funnel and, hence, was easily removed and saved. The methanol: water layer was twice 'washed' by the addition of 25 ml portions of chloroform:methanol 2:1. As with the regular extraction procedure, an appropriate amount of water was added and the flask shaken and allowed to stand 24 hours. The subnatant chloroform layer was again removed and was added to the previously collected chloroform sample. Finally, the chloroform was evaporated and the remaining lipid was resuspended in appropriate solvent for lipid fractionation upon silicic acid columns.

E. Colorimetric Micro-Determination of Oleic Acid (17).

1. Preparation of samples: As described in the section of Methods and Materials, the fatty acid fraction taken from the silicic acid-KOH column had been quantitatively transferred into a counting vial, the solvent evaporated, 15 mls scintillation fluid added, and the radioactivity measured with a Packard Tri-Carb scintillation spectrometer (Model 314EX-2). After the fatty acid samples had been counted they were prepared for a

colorimetric quantitative determination by evaporation of the scintillation fluid followed by quantitative transferal (using chloroform) of the remaining lipid into 100 ml volumetric flasks. The samples were brought to volume with chloroform.

2. Preparation of oleic acid standards: Standards were prepared by diluting oleic acid (U. S. P.; Mallinckrodt) in chloroform to known concentrations ranging from 0.02875 mg oleic acid/ml (100 μM) to 0.007187 mg oleic acid/ml (25 μM). Precautions were taken to insure that the preparation of the oleic acid standards was compatible with the preparation of the experimental samples.

3. Procedure: Five mls of the chloroform solutions of fatty acid (as prepared above) were placed in 15 ml glass-stoppered centrifuge tubes, together with 2.5 mls copper reagent. The tubes were stoppered, shaken vigorously for two minutes on a 'Vortex Junior', and centrifuged briefly in order to separate the water and chloroform phases. Following centrifugation, the supernatant aqueous phase was carefully removed by suction; all traces of the aqueous phase (containing copper) must be removed. A 3 ml portion of subnatant was pipetted into a clean, dry, stoppered test-tube, care being taken to see that the pipette did not touch the inner wall of either tube as traces of copper-containing aqueous phase might be transferred. 0.5 ml diethyldithiocarbamate reagent was added to each tube and, after the solutions had been mixed, the extinction was read at 440 mμ (Beckman spectrophotometer, Model B) in a 1 cm light-path

cuvette against a blank solution which had been subjected to the same procedure.

4. Reagents:

a) Copper reagent: 9 volumes of aqueous 1 M triethanolamine, 1 volume of 1 N acetic acid, and 10 volumes of 6.45% (w/v $Cu(NO_3)_2 \cdot 3H_2O$.

b) Diethyldithiocarbamate reagent: 0.1% (w/v) solution of Sodium diethyldithiocarbamate in 2-butanol.

c) Both reagents were made fresh every two days.

F. Preparation of Scintillation Fluid.

1. The following reagents (purchased from Packard Instrument C.) were dissolved in 2 liters of toluene.

a) 0.5 gm POPOP (1, 4-bis-2-[5-Phenyloxazolyl] - Benzene; Scintillation Grade.

b) 5.0 gms PPO (2, 5-Diphenyloxazole; Scintillation Grade).

Table 17

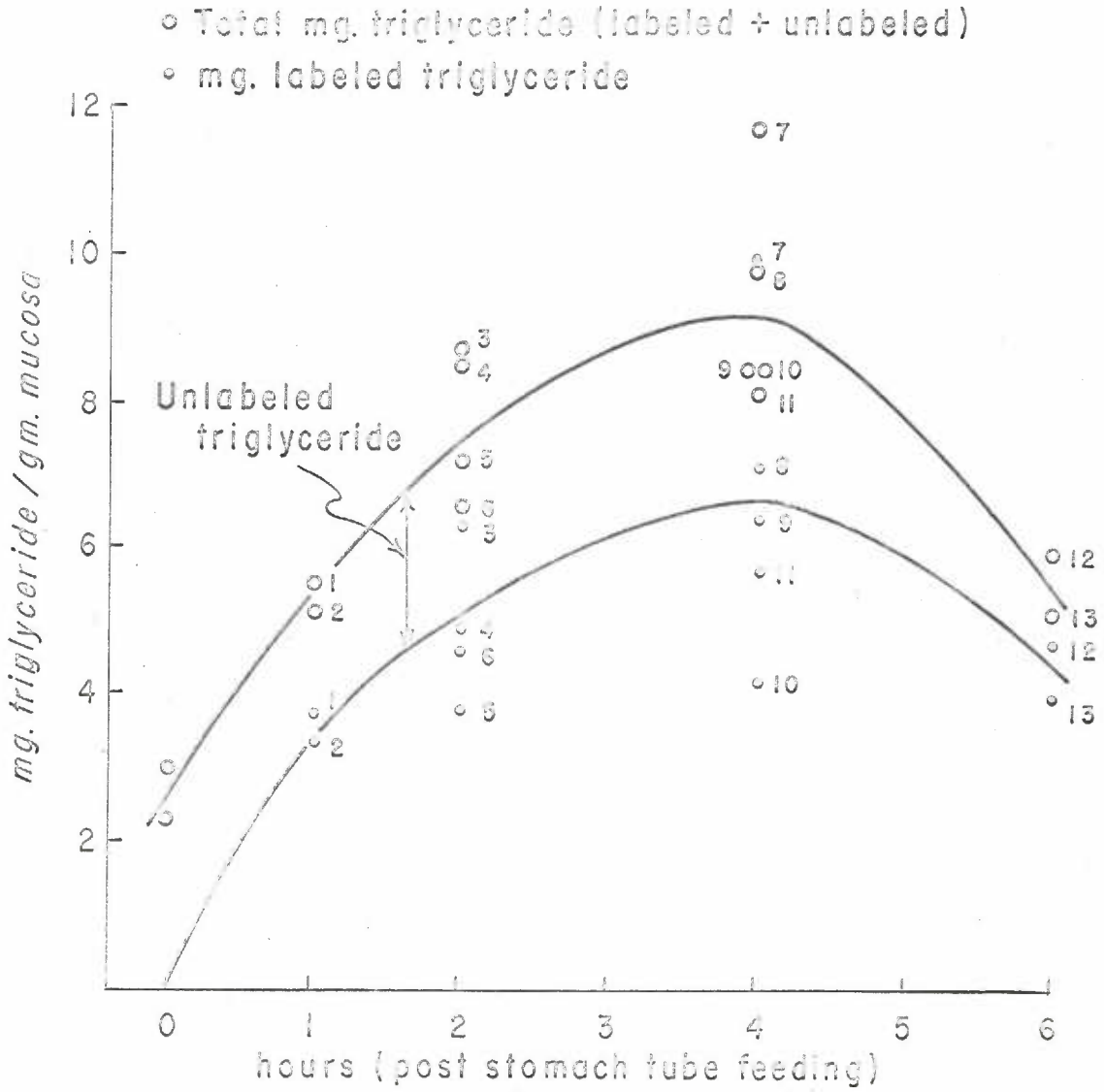
Mgs Unlabeled Fatty Acid per gram of Mucosa

in Normal and Bile Deficient Rats

Normal Rats	Hours post-prandial	Mgs unlabeled fatty acid per gram of mucosa
	0	.31
		.58
	1	.09
		.24
	2	.01
		0
		.105
		.126
	4	.104
		.167
		.156
		0
	6	.011
		.016
Bile Deficient Rats		
	1	.78
	2	.54
	4	.032
		.50
	6	0
Rats in which no absorption occurred		
	1	.30
	2	.46
	6	.37

FIGURE 12

Changes with time of total mg of triglyceride, mg of labeled triglyceride, and mg of unlabeled triglyceride in the mucosa of normal rats.


o Total mg. triglyceride (labeled + unlabeled)
o mg. labeled triglyceride
Unlabeled triglyceride
mg. triglyceride / gm. mucosa
hours (post stomach tube feeding)
0
1
2
3
4
5
6
2
4
6
8
10
12