1-1-1971

Biosynthesis of triglycerides in Mycobacterium smegmatis.

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BIOSYNTHESIS OF TRIGLYCERIDES IN *MYCOBACTERIUM SMEGMATIS*

A Dissertation Presented

By

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Submitted to the Graduate School of the
University of Massachusetts in
partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

October 1971

Major Subject: Plant and Soil Sciences

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October 1971

Dedicated to my wife,

my mother,

and the memory of my father.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Robert W. Walker for his constructive criticism and guidance throughout the course of this study. Special thanks are due to Drs. Anthony Gawienowski and Wassef Nawar, members of the thesis committee, for their suggestions and for reading this manuscript.

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INTRODUCTION

For many years lipid metabolism received little attention from biologists and biochemists. Bacteria and other microorganisms were thought to contain minor quantities of unremarkable lipid compounds which were metabolized in a manner similar to that of higher animals and plants. Recent developments in the area of lipid research showed that lipids in bacteria are different in their nature and metabolism from those of higher organisms. Furthermore, these lipid components differ within different bacterial groups to the extent that generalizations that apply to all bacteria cannot be made.

Because of the abundance of triglycerides in *Mycobacterium smegmatis* under the culturing conditions, this investigation was undertaken to study the structure, function and biosynthesis of triglycerides in this organism. An attempt at understanding the regulatory mechanisms involved in the synthesis of these compounds was also made.

LITERATURE REVIEW

Although triglycerides* (TG) are a major lipid component in mycobacteria, little is known of the structure, function or mode of formation of these cell constituents. One hypothesis regarding the function of TGs in Mycobacterium smegmatis is that they act as storage products analogous to poly-B-hydroxybutyrate and glycogen in Eubacteria. This hypothesis is based on the observation that the TG content of cells grown in carbon-rich media is high, whereas the concentration is low when cells are grown in carbon-poor media (Walker, unpublished data). This is in contrast to other generalizations that the lipid content of most bacteria usually increases with age or when the cells are under a stress (47).

Mycobacterial TGs exhibit certain unique properties which in fact reflect the uniqueness of the fatty acids (FA) that are synthesized in the cells. One such property is the presence of long-chain fatty acids (C-20 and C-24) in concentrations of 15-20% of the total TG fraction (69), which suggests that these fatty acids might be stored in the TGs at the 3-position (69), to be donated later to mycolic acids (3) and

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*Abbreviations used in this thesis: TG (triglyceride), DG (diglyceride), MG (monoglyceride), FA (fatty acid), PA (phosphatidic acid), PE (phosphatidylethanolamine), CL (cardiolipin), PIM (phosphatidyl inositol mannoside), a-GP (alpha-glycerophosphate), ACP (Acyl carrier protein), CFE (Cell-free extract).

eventually be incorporated in the cell wall (37). Tuberculostearic acid (12-methylstearic) is found in low concentrations (less than 5%) in the TG fraction. This acid is synthesized from oleic acid by receiving a methyl group from S-adenosyl methionine (SAM) probably while the oleate is attached to a phospholipid molecule (2). The fact that tuberculostearic acid is found in low concentrations in TGs suggests that there is little turnover of the acyl groups from the phospholipids to the TGs.

Biosynthesis of Fatty Acids

It was long believed that the biosynthesis of fatty acids was probably the reversal of B-oxidation. Now, however, biosynthesis has been proved to proceed via a pathway which distinctly differs from that of degradation in several ways. In biosynthesis the basic adding unit is malonyl-CoA, not acetyl-CoA which is the major "splitting unit" in degradation; NADPH (TPNH) is the reducing agent in biosynthesis not NAD (DPN); and most importantly, acyl moieties are acylated to protein (acyl carrier protein, ACP) in the biosynthetic pathway not to CoA (47).

Biosynthesis of long-chain saturated fatty acids involves two major steps:

1. Carbon dioxide fixation which produces malonyl-CoA from acetyl-CoA.
2. Elongation, where the basic adding unit is malonyl-CoA.

Although the details of the mechanisms involved in the synthesis of these fatty acids were arrived at from working with mammalian and avian systems, there is extensive evidence that the malonyl-CoA pathway

occurs widely in bacteria (33, 39, 48).

In mammalian cells, CO_2 fixation occurs in what is known as the non-mitochondrial fraction, whereas the elongation sequence takes place in the mitochondria. In microorganisms the two processes probably take place in the membrane system. The sequence of steps involved in long-chain saturated fatty acid synthesis, the details of which have been worked out mainly through the work of Vagelos (62) and Wakil (68), are the following:

$$HCO_3^- + ATP + \text{biotin-enzyme} \longrightarrow CO_2\text{-biotin-enz} + ADP + P_i$$

$$CO_2\text{-biotin-enz} + \text{Ac-CoA} \longrightarrow \text{Biotin-enz} + \text{Mal-CoA}$$

The enzyme, carboxylase, requires both biotin and Mn^{++} ions for the catalysis of the reaction.

Elongation

After the acetyl- and malonyl-CoA derivatives are formed, the acyl groups are transferred to ACP through the catalytic action of acetyl and malonyl transferases to yield acetyl-ACP and malonyl-ACP respectively. The produced compounds condense together to form a 4-carbon compound, acetoacetyl-ACP, with a concomitant release of one molecule of CO_2. Acetoacetyl-ACP produces butyryl-ACP through a series of reactions involving reduction, oxidation and dehydration. Butyryl-ACP then condenses with another malonyl-ACP to give rise to a 6-carbon fragment, and so on until palmitic acid is formed. The first carbons of palmitic acid are derived from acetyl-ACP and the rest of the carbons from malonyl-ACP. The net reaction is:

$$1\ \text{Ac-ACP} + 7\ \text{mal-ACP} + 14\ \text{NADPH} + 14\ H^{+} \longrightarrow$$

$$1\ \text{palmitate} + 7\ CO_2 + 8\ \text{ACP} + 14\ \text{NADP} + 6\ H_2O$$

One important factor in this pathway is ACP which is now recognized to play a major role in lipid biosynthesis. It is a very stable protein (withstands temperatures of 90°C for 5 minutes), and has a molecular weight of approximately 10,000. Its functional group is very similar to that of CoA (39).

ACP has been isolated and purified from E. coli (41), Arthrobacter viscosus (58), Clostridium butyricum (1) and Mycobacterium phlei (44, 45). Matsumura et. al. (44) have found that $ACP_{M.\ phlei}$ differs from other bacterial ACP in its content of proline per mole of protein (4 moles proline / mole protein in contrast to one proline residue / mole protein in $ACP_{E.\ coli}$). Furthermore, Matsumura (45) found that $ACP_{M.\ phlei}$ supports the malonyl-CoA-CO_2 exchange reaction in E. coli extracts with an activity about half that of $ACP_{E.\ coli}$. $ACP_{M.\ phlei}$, however, was found to inhibit the fatty acid synthatase system of E. coli. $ACP_{E.\ coli}$ and $ACP_{A.\ viscosus}$, on the other hand, were functionally indistinguishable (58).

ACPs from different bacteria differ in their location in the cell. $ACP_{E.\ coli}$ was found to be located in the plasma membrane or very near to the cytoplasmic side of the membrane and can be easily isolated (64). $ACP_{M.\ phlei}$ on the other hand is located in a particle free extract but is found in close association with a multienzyme complex (fatty acid synthetase) (9). This multienzyme complex is dissociated in solutions of low ionic strength.

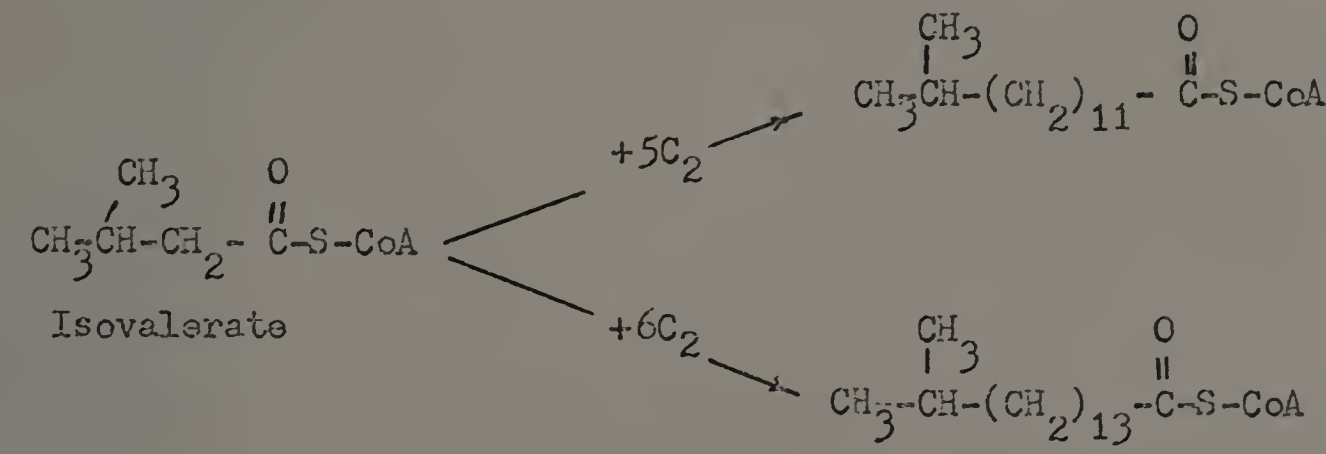
One other way through which bacteria can make certain long-chain saturated fatty acids is the hydrogenation of unsaturated fatty acids. This activity has been studied in certain rumen bacteria (52). The main fatty acid converted by biohydrogenation to saturated fatty acids is linolenic acid which is introduced into the rumen in the diets of ruminants. This is converted in a step-wise fashion into linoleic, oleic and finally stearic (70).

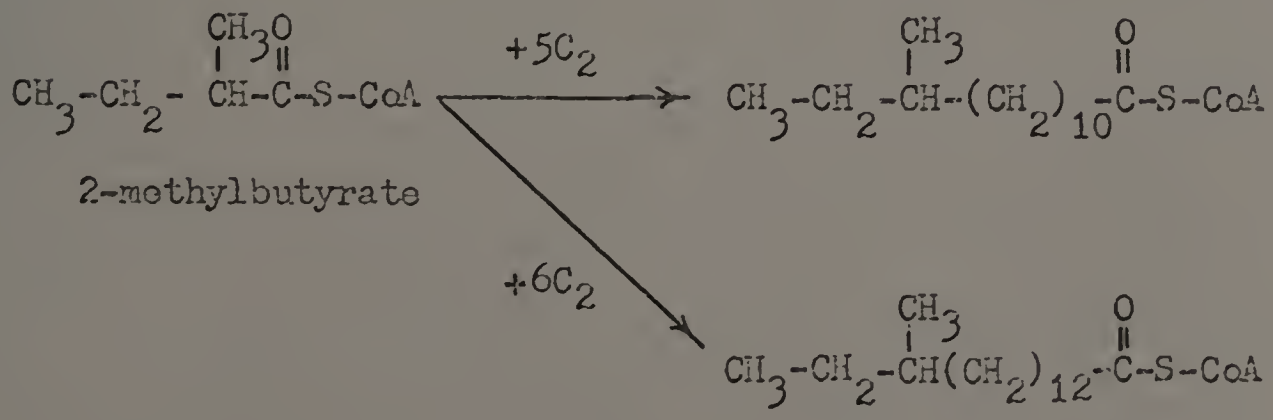
Generally, microorganisms synthesize unsaturated fatty acids in two major ways (32, 47): elongation of unsaturated short-chain intermediates, and dehydrogenation of long-chain saturated fatty acids. In mycobacteria, biosynthesis of unsaturated FAs has been studied in relation to specific reactions: desaturation of stearate to oleate and palmitate to ^{10}-hexadecanoate (24); the condensation reactions of long-chain keto, hydroxy and unsaturated FAs (stearate and tetraacosanoate) to form the C_{60} and C_{80} of mycolic acids (2). Hung and Walker (25) showed that the long-chain unsaturated FAs of M. smegmatis and M. bovis BCG are related and reflect a common origin from the ^{9}- C_{18} fatty acid, with two-carbon elongations. These authors suggest that long-chain FAs (C-20, C-22, C-24) might arise by a combination of desaturation and elongation together.

In addition to the saturated and unsaturated fatty acids, there are groups of fatty acids that are unique and characteristic of certain bacteria. Such FAs include the cyclopropane FAs, branched FAs and certain FAs characteristic of one group of organisms such as corynemycolic acids in Corynebacterium diphtheria and smegmamycolic acid in M.

smegmatis. The cyclopropane FAs and some branched FAs derive their extra carbon unit from SAM (2, 38) in order to form the cyclopropane ring or branching unit. It was found that the carbon and two of the hydrogens are donated from SAM whereas the third hydrogen comes from another source (50, 51). Neither the reason nor the significance of such a mechanism is understood.

Other branched fatty acids arise either from isovalerate (derived from leucine) or 2-methylbutyrate (derived from isoleucine) and are elongated in this fashion:





The elongation mechanism involved in these pathways is the malonate scheme (33). The fatty acid intermediates involved in the branching process are ACP esters rather than CoA esters (47).

Biosynthesis of Triglycerides

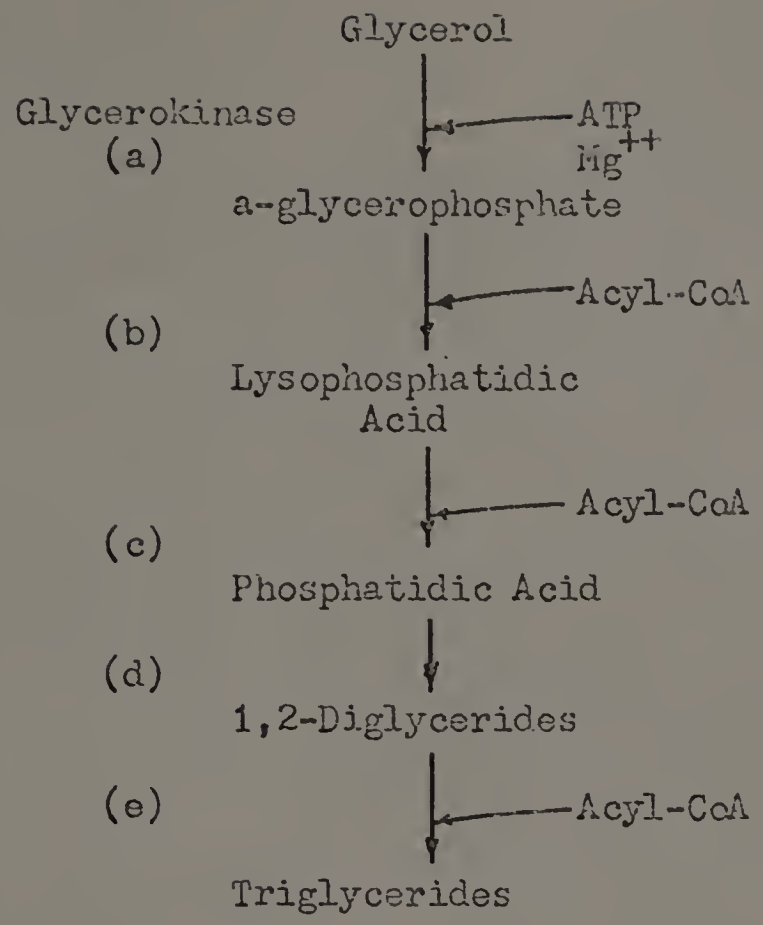
In mammalian systems, and prior to the esterification of the different fatty acids to glycerol, the fatty acids have to be converted to acyl-CoA thioesters by the action of various thiokinases (19, 23, 41). Three different kinases catalyzing the phosphorylation of fatty acids in relation to their chain length have been identified. The general reaction proposed by Berg (5) for such a process is:

$$R\text{-}C\text{-}CO^{-} + ATP \longrightarrow R\text{-}CO\text{-}AMP + P\text{-}O\text{-}P$$

$$R\text{-}CO\text{-}AMP + CoASH \longrightarrow R\text{-}CO\text{-}S\text{-}CoA + AMP$$

A similar mechanism operates in bacteria for the activation of the fatty acids. However, another activation step involving the transacylation of the acyl moiety from acyl-CoA to ACP was found to occur in bacterial systems (62).

Tietz and Shapiro (61) were among the first investigators to study the mechanisms of triglyceride synthesis in mammalian systems. They found that ATP was required for the incorporation of FAs into the triglycerides of rat liver homogenates. One pathway proposed for the synthesis of TGs in the liver is the a-glycerophosphate pathway:



Reaction (a) is catalyzed by glycerokinase and requires ATP and magnesium ions. The reactions of a-GP with acyl-CoA (reactions (b) and (c)) have been studied by Lands et. al. (35, 36). These workers used guinea pig liver microsomes to form diacylglycerophosphate (PA) from glycerophosphate and CoA esters of stearate and linoleate. Lysophosphatidic acid did not accumulate as an intermediate since it was very rapidly acylated to form PA. The two acylation reactions are controlled by different enzymes. The first acylation was found to be sensitive to SH inhibitors but the second acylation was not.

Reaction (d) is catalyzed by PA-phosophatase (59) to yield DG and P_i. The DG is then esterified with acyl-CoA to yield TG (reaction (e)).

A pathway similar to the a-GP pathway seems to operate in bacterial lipid synthesis. Evidence for the existence of lyso-PA and PA as intermediates was obtained when E. coli mutants defective in the acylation of

a-GP (14) and lyso-PA (22) were isolated. Early work done by Goldfine et. al. (18) demonstrated the involvement of ACP in the pathway. These workers showed that the conversion of a-GP to lyso-PA, in the presence of a particulate fraction from Clostridium butyricum, was dependent on the addition of ACP isolated from C. butyricum or E. coli. Moreover, chemically synthesized ^{3}H-palmityl-ACP stimulated the acylation of a-GP in the same particular fraction. Recent work (65) with E. coli, however, proved that the first acylation of a-GP requires either acyl-CoA or acyl-ACP as donors of the acyl group, but synthesis of PA from lyso-PA requires acyl-ACP.

Reports of the synthesis of phosphatidylserine and phosphatidylglycerol (30, 34) in E. coli and other bacteria suggest that PA (in the coenzyme form, CDP-DG) is an important precursor of bacterial lipids. This prompted a study by Pieringer and Kunnes (49) of the biosynthesis of PA in E. coli. These workers found that the particulate fractions of E. coli catalyze the phosphorylation of DG by ATP to form PA in a typical phosphorylation reaction:

$$\text{ATP} + \text{1,2-dipalmitin} \xrightarrow[Mg^{++}]{\text{Kinase}} \text{PA} + \text{ADP}$$

The same particulate fraction was found to catalyze the synthesis of lyso-PA from monopalmitin.

It is thought that CDP-DG is the obligatory intermediate in the de novo synthesis of all lipids in E. coli. Chang and Kennedy (11) have shown by pulse-labeling experiments in intact E. coli that PA is rapidly formed and has a high rate of turnover. Presumably, it serves as a

precursor for other lipids by first reacting with CTP to form CDP-DG (11):

$$PA + CTP \longrightarrow CDP\text{-}DG + P_i$$

Carter (10) described an enzyme in the membrane fractions of E. coli that catalyzes the above reaction. Hutchinson and Cronan (27) and McCaman and Finnerty (46) demonstrated the synthesis of CDP-DG in the membrane fraction of Saccharomyces cerivisiae and Micrococcus cerificans respectively.

Another pathway that is operative in TG synthesis in mammalian systems is the monoglyceride pathway. This pathway was shown to operate in rat mucosa (28) and adipose tissue (29). In 1960, Clark and Hubscher (12, 24) provided the first evidence for the biosynthesis of TGs from monoglycerides. These authors demonstrated that the addition of MGs markedly stimulated the incorporation of ^{14}C-palmitic acid into TGs under conditions where the a-GP pathway was inhibited. Senior and Isselbacher (57) further showed that ^{14}C-glycerol-labeled monopalmitin was incorporated into DGs and TGs. The final confirmation of the existence of the MG pathway was provided by an experiment where a doubly labeled MG (^{3}H-glycerol and ^{14}C-fatty acid) was incorporated intact into DGs and TGs (28). The steps involved in this pathway are the following:

$$\text{FA} + \text{CoA} \xrightarrow{\text{Acyl-CoA synthetase}} \text{Acyl-CoA}$$

$$\text{Acyl-CoA} + \text{MG} \xrightarrow{\text{MG transacylase}} \text{DG}$$

$$\text{Acyl-CoA} + \text{DG} \xrightarrow{\text{DG transacylase}} \text{TG}$$

Rao and Johnston (54) have purified the enzymes involved in the MG pathway from hamster intestinal mucosa. During the process of purification, acyl-CoA synthetase, MG transacylase and DG transacylase were purified simultaneously, suggesting that the enzymes of the MG pathway are grouped in a multienzyme complex. This enzyme complex has been termed "triglyceride synthetase". Other findings suggest that these enzymes are confined to the membrane fraction of the endoplasmic reticulum.

The intermediate common to the a-GP pathway and the MG pathway is 1,2-DG. Since both pathways occur in the microsomal fraction of mammalian cells, the question as to whether or not the 1,2-DGs synthesized by each pathway equilibriate was examined by Johnston (28). He concluded that the DGs formed by the two pathways do not equilibriate and that only the DGs synthesized via the a-GP pathway are precursors of more complex phosphplipids (such as phosphatidyl choline). Recent work by Johnston et. al. (29) showed that the DGs synthesized by both pathways have a 1,2- configuration but differ in the nature of the fatty acids esterified to the DGs.

The different lipids isolated from M. smegmatis show the same specificity in the nature and distribution of the fatty acids. In a recent study aimed at investigating the positional distribution of FAs in the phospholipids and triglycerides of M. smegmatis and M. bovis BCG, Walker et. al. (69) showed that these lipids of both species are asymmetric molecules. These authors showed that the alpha carbon of glycerol is predominantly esterified with $C_{18:1}$ or a derivative of it (C_{19}-branched) and the beta carbon is esterified with $C_{16:0}$ or a derivative of it, (C_{17}-branched) in the TGs and phospholipids of M. smegmatis. This may suggest that both the TGs and phospholipids of M. smegmatis may be derived from a common intermediate.

Regulation and Control

Like any other cell component, lipid synthesis and degradation are governed by the different physiolcgical states that the cell may pass through such as availability of nutrients, enzyme induction or repression, age and the accumulation of toxic products, etc.

Marinetti et. al. (43, 16) have investigated the control of lipid synthesis in the liver by using ^{14}C-glycerol as a marker and examining the effects of varying concentrations of ATP, CTP, and Mg^{++} ions. They found that the optimal concentration of ATP and Mg^{++} ions for lipogenesis was 10 mM. Increasing ATP concentration to 100 mM eliminated labeling of all lipids except an unidentified acidic phospholipid which was suspected to be phosphatidyl glycerol phosphate.

Although ATP was found to be required for labeling all the lipids, the pattern of lipid labeling was influenced by Mg^{++} and CTP. Increasing

the magnesium ion concentration to 10 mM caused the labeling to appear mainly in the phospholipids, especially lecithin. This was done at the expense of labeling the TGs. However, increasing the CTP concentration to 10 mM, in the presence of both ATP and magnesium, shifted the labeling in favor of the di- and triglycerides.

More recently, Possmayer and Hudd (53) showed that the presence of CMP, CDP or CTP markedly decreased the incorporation of ^{14}C-glycerol-3-phosphate into phospholipids but had no effect on glycerides in rat brain. The decrease in the incorporation of glycerol-3-phosphate into phospholipids was related to a decrease in the synthesis of PA. These results indicate that cytidine nucleotides, particularly CTP, can modify the acylation of a-GP.

Other regulatory mechanisms of TG synthesis in higher organisms have been ascribed to the concentration of acetyl-CoA and citrate in the tissue and of possible effectors of acetyl-CoA carboxylase (long-chain acyl-CoA as an inhibitor, (7) and citrate as an activator (63)). In one study, Denton and Halperin (15) have shown that the rate of TG synthesis could not be correlated with the concentration of either glycerol-3-phosphate or long-chain fatty acyl-CoA in rat adipose tissue. In addition, no correlation was observed between the rate of FA synthesis and to whole-tissue concentration of acety-CoA, citrate or acetyl-CoA synthetase. They suggest that the control of fatty acid and triglyceride synthesis resides in some additional factor,

It is evident from the above that the details of the control and regulation of TG synthesis are not well worked out yet in either the higher organisms or the bacteria.

MATERIALS AND METHODS

1. Organism. Mycobacterium smegmatis ATCC 19420 was used in this study. The organism was usually grown in Youman's medium and its growth curve was determined as follows:

Ten mls of the cell suspension was ultrasonicated for 15 seconds to break cell clumps. Turbidity of the homogeneous suspension was read in a Klett-Summerson Colorimeter (Red filter, Ca 660 mu) (Klett Manufacturing Company, New York). The generation time as determined from the growth curve (Figure 1) is approximately 9 hours.

2. Media.

a. Modified Youman's Medium.

Unless indicated otherwise, the growth medium used during the course of this study for culturing M. smegmatis was modified Youman's medium (60). The composition of the medium is:

	Per Liter
KH_2PO_4	5 g
Glycerol	40 g
Asparagine	5 g
Sodium Citrate	2.5 g
Ferric Ammonium Citrate	0.05 g

0.5 g Magnesium Sulfate was added after adjusting the pH to 7.0.

b. Kaneshiro and Marr's medium (31) was used when a salt medium was required for growth. Its composition is:

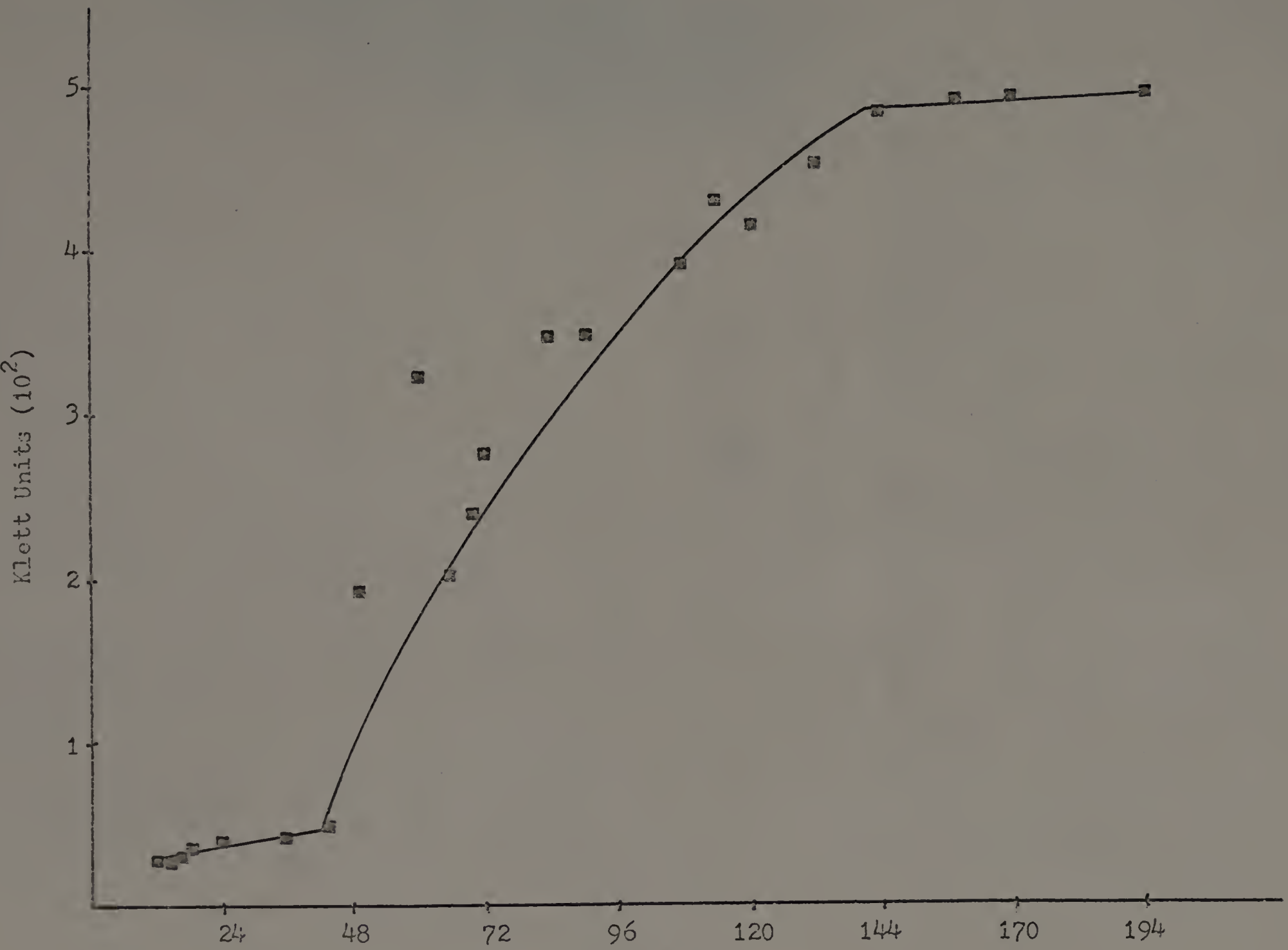


Figure 1

Growth Curve of Mycobacterium Smegmatis

	Per Liter
KCl	1.00 g
K_2HPO_4	12.20 g
KH_2PO_4	9.40 g
$(NH_4)_2SO_4$	1.50 g
$MgCl_2.6H_2O$	0.64 g
$CaCl_2.2H_2O$	0.02 g

Both $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$ were added after the pH was adjusted to 7.0.

Glucose	10.00 g

Sterilized and added separately.

c. Basal medium (21):

This medium was used in certain experiments. Its composition is as follows:

	Moles Per Liter
NaCl	8×10^{-2}
$FeCl_2$	2×10^{-6}
KCl	2×10^{-2}
Tris-HCl (pH 7.5)	1.2×10^{-1}
NH_4Cl	2×10^{-2}
Glucose	1.2×10^{-2}
$MgCl_2$	1×10^{-3}
a-glycerophosphate	1.4×10^{-4}
Na_2SO_4	5×10^{-4}
Bacto-peptone	0.04%

3. Materials. The radioisotopes were purchased from Searle-Amersham, ICN, Tracer Laboratories or Applied Science. ^{14}C-24 fatty acid was prepared in this laboratory and had a specific activity of 1 mCi/mM. Other compounds were of reagent grade purchased from Fisher, Calbiochem or P-L Laboratories. Lipid standards were products of Hormel Institute (Austin, Minnesota). Analytical grade glycerokinase was purchased from Calbiochem. It was prepared from Candida mycoderma and had a specific activity of 83 EU per mg protein.

4. Preparation of Cell-Free Extract. Cells were collected by centrifugation at 12,000 x g at the desired stage of growth, usually 72 hours. The pellet was then resuspended in phosphate buffer (0.05 M; pH 7.4) which contains dithiothreitol (10^{-3}M). The mixture was ultrasonicated for two minutes at full speed in order to suspend the cells homogeneously in the buffer. The cells were then disrupted by passing them twice through a French pressure cell (American Instrument Company, Silver Springs, Maryland) at 20,000 psi. Unbroken cells and cell debris (large cell fragments) were removed by centrifugation at 5,000 x g for 15 minutes. The supernatant solution was recovered and its protein concentration determined.

5. Determination of Protein Concentration. Protein concentration was determined by the biuret method (55). One ml of the sample was mixed with 4 mls biuret reagent (one liter solution contains 1.5 g $CuSO_4 \cdot 5H_2O$, 6.0 g $NaKC_4H_4O_6$ - sodium potassium tartarate - 10% carbonate free NaOH stored in a polyethylene bottle). After incubation for 30 minutes at

room temperature, the optical density of the mixture was read at 550 nm. The protein content of the sample was determined by comparing its optical density with a standard curve obtained by using bovine serum albumin.

6. Preparation of Crude ACP. Cell free extract was prepared from cells grown in 600 mls Youman's medium. The cell free extract was centrifuged at 5,000 x g for 10 minutes. The supernatant fraction was recovered, heated at 90°C for 5 minutes then cooled in ice. The mixture was centrifuged at 16,000 x g for 15 minutes and the pellet discarded. The supernatant fraction was then precipitated with 70% ammonium sulfate, centrifuged and the supernatant fraction discarded. The pellet was dialyzed against 5 liters of buffer (containing mercaptoethanol) overnight. The dialysate was diluted to 150 mls with distilled water. This fraction was centrifuged at 20,000 x g for 10 minutes. The supernatant solution was recovered and freeze-dried. The protein content of the preparation as determined by the biuret method was 272 mgs of a total of 896 mgs.

7. Thin Layer Chromatography. Plates were prepared by suspending the gel in water and then spreading the slurry on 20 x 20 cms plates to the desired thickness. The plates were activated at 120°C for one and a half hours. For the isolation of neutral lipids, Adsorbosil-1 ($CaSO_4$ binder) (Applied Science Laboratories, Inc., University Park, Pennsylvania) was used whereas Adsorbosil-3 ($MgSiO_4$ binder) (same source as Adsorbosil-1) was used for isolating polar lipids. The neutral lipids were separated

by spotting the sample and a standard about one inch from the bottom of the plate and developing it in one of these solvent systems: ether:benzene:ethanol:acetic acid (40:50:2:0.2) (17); or hexane:ether: acetic acid (70:20:7). Both systems gave good separation of the neutral lipids which were located by comparison with the standard after spraying the plate with rhodamine 6-G and viewing it under ultra violet light.

The phospholipids were separated on Adsorbosil-3 plates by developing the plates in one of these solvent systems: chloroform:methanol: water (65:25:4), drying the plate for one hour, then redeveloping in petroleum ether:ether:acetic acid (80:20:2); or chloroform:methanol: acetic acid:water (80:20:8:0.3), drying then developing in ether:acetic acid (95:5). Along with the sample, a standard was routinely spotted. The standard was sprayed with Vaskovsky and Kostetsky spray (66) (phospholipids turn blue) and the rest of the plate was sprayed with rhodamine 6-G. Bands of the sample that corresponded to the standard were located by examining the plate under UV.

8. Measurement of Glycerol Utilization. In order to determine the amount of glycerol utilized, proper dilution of the growth medium was first performed. To a two ml sample of the medium (containing between 3 and 5 ug glycerol after dilution), 0.1 ml of 10 N H_2SO_4 and 0.5 ml of 0.1 M sodium periodate were added. The mixture was left to stand for 5 minutes at room temperature, then 0.5 ml of 10% sodium bisulfite was added. One ml of the above solution was then mixed with 5 mls of chromotropic acid solution (1% chromotropic acid to which 450 mls 4 N H_2SO_4 was added). The tube was then heated for 30 minutes in a

water bath, cooled to room temperature, then 0.5 mls of 5% thiourea (half saturated solution) was added to remove the dark color. The optical density was then read at 570 nm and the concentration of glycerol determined by comparison with a standard curve.

9. Determination of Triglyceride Content of the Cells. Cells were grown in Youman's medium under varying conditions--time, glycerol concentration in the medium, etc.--collected by centrifugation then freeze dried. Fifty mgs of the freeze-dried cells were ultrasonicated for 5 minutes in 15 mls of chloroform:methanol (2:1) and then centrifuged at 15,000 x g. The solvent was collected, the pellet resuspended in the same solvent, and the mixture centrifuged again. The solvent fractions were pooled, then the solvent was evaporated under reduced pressure. A measured sample of the total lipid fraction was then streaked on Adsorbosil-1 plates and the triglycerides located as previously described. The TG band was collected in tubes containing 15 mls chloroform:methanol (80:10), centrifuged, and the solvent fraction recovered. This procedure was repeated twice to insure complete recovery of the TGs, and the TGs were weighed on a microbalance. This procedure was found to give 99.5% recovery of a predetermined amount of TGs.

10. Measurement of Radioactivity. Radioactivity measurements were performed by using a 2-channel liquid scintillation counter. Five-Ten mls modified Bray's solution (8) were added to a scintillation vial containing the sample and the mixture thoroughly mixed. The modified Bray's solution consists of the following:

	Per Liter
Naphthalene	60 g
2,5 Diphenyloxazole (PPO)	4 g
P-BIS [2-(5-phenyloxazolyl)] - Benzene (POPOP)	0.2 g
Methanol	100 mls
Ethylene glycol	20 mls
Toluene	100 mls

Dioxane was added to make 1 liter.

When the different lipid classes were separated on TLC, they were scraped from the plates into scintillation vials and counted. There was little interference from the gel with counting as determined by control experiments where known amounts of labeled lipid were spotted on plates, scraped, and counted.

11. Preparation of Doubly Labeled Monoglyceride. Doubly labeled monoglyceride was prepared according to the method of Hartman (20) with some modification. The procedure was as follows:

0.5 gm of glycerol and 500 uCi ^{3}H-glycerol (Sp. Act= 420 mCi/mM) were refluxed with 3.0 mls acetone, 4.0 mls alcohol-free chloroform, and 0.015 gm toluene-p-sulfonic acid (catalyst for the reaction) for 100 minutes. Five hundred mgs palmitic acid and 100 uCi ^{14}C-palmitic acid (Sp. Act. 5mCi/mM) were then added, and the mixture was allowed to reflux for an additional 3 hour period. After this time, the catalyst was removed by adding 0.05 gm anhydrous sodium acetate. The mixture was then washed 3 times with water, and the chloroform was evaporated under

reduced pressure. Scission of the isopropylidene-glycerol-palmitate was done by dissolving the ester in 12 mls of 2-methoxyethanol and heating with finely powdered boric acid (1g) on a boiling water bath for 30-40 minutes. The mixture was then taken up in ether, washed three times with water, and dried over Na_2SO_4.

The precipitate was then dissolved in 20 mls hexane:ether (3:1 v/v) and applied to a 3.1 x 20 cm column of Florisil (Fisher Scientific Company, Fair Lawn, New Jersey) which had been deactivated with 7% (w/w) added water. DGs and TGs were eluted with 300 mls (2 column volumes) hexane:ether (3:1). The MG was eluted with 400 mls of ether containing 2% methanol (42). The MG gave one spot by TLC. Ten ml of the synthesized MG was counted in the scintillation counter set up to count two isotopes at once. The ratio of ^3H:^{14}C in the MG was determined.

12. Experiments with Doubly Labeled MG. Crude cell free extract was prepared from young cells grown in Youman's medium. Half a ml of the extract was incubated with the following:

0.75 ml buffer (0.05M Phosphate buffer containing 10^{-3}M DTT; pH 7.4)

25 umoles NaF

10 umoles $MgCl_2$

1 u mole palmityl-CoA

1 u mole doubly labeled monopalmitin suspended in 0.1 ml of 10% Tween-80.

The monoglyceride had total counts of 74,280 cpm ^3H and 211,540 cpm ^{14}C. The reaction mixture was identical to that used by Johnston (28), who showed the existence of the MG pathway in mammalian systems.

The reaction tube was shaken on the Mickle shaker for 30 seconds to allow for complete homogeneity of the contents. The reaction, which was allowed to proceed for 60 minutes, was stopped by the addition of 3 mls of chloroform:methanol (2:1). The tube was shaken vigorously and centrifuged for 15 minutes. The chloroform layer containing the lipids was carefully removed and the solvent evaporated under a stream of nitrogen. The lipids were dissolved in 100 ul chloroform. Fifty ul was spotted on Adsorbosil-1 plates and developed in hexane:ether:acetic acid (70:20:7). The remaining 50 ul of the sample was spotted on Adsorbosil-3 and developed in chloroform:methanol:acetic acid:water (80:20:8:0.3), dried for 1 hour, then redeveloped in ether:acetic acid (95:5). The neutral and phospholipids were isolated and counted.

In another experiment the cell free extract was fractionated into pellet and supernatant fractions by centrifugation at 40,000 x g for 1 hour. Both fractions were retained and used in the following incubation mixture:

1.00 ml of pellet or supernatant

0.60 ml Buffer

2 umoles doubly labeled monoglyceride

5 umoles oleic acid

5 umoles ATP

0.2 umoles CoA

20 umoles $MgCl_2$

The monopalmitin used in this experiment was a mixture of the chemically synthesized monopalmitin and monoglyceride isolated from

cells after they had been incubated with ^{3}H-glycerol and ^{14}C-palmitic acid. This preparation had a ratio of $^{3}H/^{14}C$ of 0.3 (10 ul sample had ^{3}H 245 cpm and ^{14}C 738 cpm).

The procedure and conditions used for this experiment were identical to the previous one with doubly labeled monopalmitin.

13. Starvation Experiment. Three-day old cells which had been grown in Youman's medium were aseptically harvested and washed twice with sterile distilled water. The cell paste was resuspended in the salt medium (Kaneshiro and Marr's medium without glucose) containing 12.26 uCi ^{14}C-palmitic acid (Sp. activity= 17.4 mCi/mM) by shaking. The flask was incubated at 26°C for 30 minutes. The cells were harvested aseptically and washed twice with sterile distilled water, and then were resuspended in the salt medium without palmitic acid. After complete suspension in the new medium, an 8 mls sample was mixed with chloroform:methanol in order to make solvent ratios of 1:2:0.8 (C:M:H_2O). The cells were left in the above extractant for 1 hour, then more chloroform and water were added to make the final chloroform:methanol:water ratio 2:2:1.8. The suspension was left at room temperature overnight to allow for the chloroform layer to separate completely from the aqueous layer. The chloroform layer was then removed, filtered to remove cell debris, and dried. The same procedure was repeated with cells incubating in the salt medium after 12, 24, and 36 hours. The lipids were then isolated and counted for radioactivity.

14. Pulse-Labelling Whole Cells. Three-day old cells, grown in 100 mls Youman's medium, were harvested by centrifugation and resuspended in 50 mls basal medium. To this suspension, 5 uCi ^{14}C-glycerol (Sp. Act=20 mCi/ml) were added, and the flask was incubated at room temperature for 5 minutes with shaking. The cells were then collected by millipore filtration and were washed once with 20 mls basal medium. Over a clean flask, the cells were washed again with 30 mls cold distilled water. This procedure has been reported (21) to leach small molecules from bacterial cells and has been observed to release more than 95% of the cold-trichloroacetic acid-soluble fraction. The filtrate was then evaporated under vacuum and then applied to Whatman number 1 filter paper along with glycerol and a-glycerophosphate standards. The filter paper was developed (descending) in methyl-cellosolve:methyl ethyl ketone: 3N NH_4OH (7:2:3: v/v) overnight (21). The standards were located by using the following sprays: a-glycerophosphate: Haynes-Isherwood (4) indicated GP as a blue spot over a white background. Glycerol: the part of the filter paper containing glycerol was sprayed with 0.5% $NaIO_4$ until it became damp. The paper was allowed to stand for 5 minutes then was sprayed with 0.5% benzidine in ethanol:acetic acid (4:1) (6). Glycerol appears as a white spot over a dark gray background.

The region of the filter paper where the sample was applied that correspond to the a-GP spot was cut in small pieces and directly counted in Bray's solution.

The filter paper with the cells on it was suspended in 100 ml chloroform:methanol (2:1) overnight. The suspension was then filtered

and the solvent removed by evaporation under vacuum. The lipids were dissolved in 2 mls Folch solvent (C:M:H_2O; 86:14:1) and spotted on plates. The neutral and phospholipids were scraped from the plates and counted.

15. Pulse Labelling with 2 Isotopes. Three-day old cells grown in 100 ml Youman's medium were incubated with the following:

a. 12.5 mgs palmitic acid suspended in 2.5 ml of 5% bovine serum albumin and 2.4 uCi ^{14}C-palmitic acid (Sp. Act. 17.4 mCi/mM)

b. 5 uCi ^{3}H-glycerol (Sp. Act. 420 mCi/mM) (lots of glycerol in the medium).

The suspension was incubated at room temperature with shaking. Five ml aliquots were removed after 1, 5, 10, 30, and 60 minutes of incubation with the isotopes. The aliquots were filtered through Whatman No. 5 filter paper and washed with 10 mls cold distilled water. The filter paper was then transferred into 30 ml C:M (2:1). The next day the mixture was filtered, and the solvent evaporated. The lipids were taken up in 5 mls ether and transferred to another vial. After the ether was evaporated the lipids were dissolved in 100 ul Folch solvent and spotted on TLC plates. The different lipids were located, scraped, and counted.

16. Pulse-Chase Experiment. Cells from 100 mls Youman's medium were collected after 3 days of growth, washed with KM medium without glucose, and then resuspended homogeneously in 50 mls of the same medium. To the new suspension, 100 uCi ^{3}H-glycerol (Sp. Act. 420 mCi/mM) was added, and

the mixture was left to incubate at room temperature for 20 minutes with shaking. Eight mls of this suspension were then mixed with chloroform: methanol in order to make solvent ratios of 1:2:0.8 ($C:M:H_2O$). More chloroform:water was added after one hour to make the final $C:M:H_2O$ ratio equal 2:2:1.8. The suspension was left at room temperature overnight, then the chloroform layer was removed and filtered. The solvent was evaporated and the lipids isolated and counted.

The rest of the sample was centrifuged, and the cells were washed twice with the salt medium. The pellet was resuspended in Youman's medium containing 1% glycerol to chase the label. Eight ml samples were taken at 0, 10, 30, and 60 minute intervals and were treated as the previous sample. The lipids were isolated and counted.

17. Assay and Inhibitors of ATPase. The assay method for ATPase in M. smegmatis is a modification of that reported in Methods of Enzymology (40). The assay procedure involves the measurement of inorganic phosphate that is liberated from ATP when it reacts with molybdate in an acidic medium.

Equal parts of 5 mM ATP and buffer (Tris, 0.03 M; 2-amino-2-methyl-1,3-propandiol, 0.03M; HCl, 0.03 M; $MgCl_2$, 2 mM) were mixed. In an ice bath, 25 l cell free extract and 100 ul of the ATP/buffer mixture were added. The test tubes were then incubated at 38°C for 30 minutes. The reaction was stopped with 20 ul of 30% TCA. To each test tube, 2 mls of freshly mixed phosphate reagent [containing 2 mls of 2.5% ammonium molybdate, 46 ul acetate buffer (0.1 ml acetic acid and 0.065 M sodium acetate); 2 mls 1% ascorbic acid] were added and allowed to react for 15

minutes at room temperature. The tubes were centrifuged, and the optical density read at 870 nm. The blank was a test tube containing the above reagents at 0 time. Each tube contained 5 25 ul of 15 mM phosphate as an internal standard.

Either nickel or fluoride ions were included in the assay mixture in order to examine the effect to these ions on ATPase. Both ions were reported as inhibitors of the enzyme (13).

18. Experiments with Cell Free Extract. In order to elucidate the pathways in TG synthesis, the majority of the experiments were done with a cell free system. In most of these experiments, one general procedure was used. The cells were broken and a cell free extract which contained between 10-20 mgs protein/ml was prepared without any further fractionation, unless indicated otherwise.

Since all the experiments with the CFE involve acqueous systems, water-insoluble material that was concurrently used was solubilized by attaching it to albumin. Thus when long-chain fatty acids were used, they were either ultrasonicated or shaken with a relative amount of 5% bovine serum albumin (depending on how much FA was used) on a Mickle shaker for one minute at full speed, then adjusted to a pH of 8.5-9. These procedures were found to be satisfactory for suspending the fatty acids in water.

The reaction mixture which allowed acceptable incorporation of ^{14}C-fatty acids in the different lipid classes contained, in addition to the cell free extract, CoA, ATP, a-GP, Mg^{++} and Ni^{++} ions. In order to optimize the conditions necessary for best incorporation of fatty acids

into neutral and phospholipids, the concentrations of the above-mentioned factors were varied. Further experiments on the effect of incubation time, GSH and CTP concentration, were performed.

19. Lipase Hydrolysis of TGs. In these experiments the crude cell free extract was incubated with the necessary factors required for optimal TG synthesis, but the reaction was scaled up 5 times. After the reaction was allowed to proceed for 60 minutes, the synthesized TGs, labeled with the added ^{14}C-fatty acids, were scraped from the plates and eluted from the gel with C:M (80:10). The collected TGs (3 mgs) were then incubated with 1 ml Tris buffer (0.5M pH 7.45), 0.2 ml 22% $CaCl_2$, 0.3 ml 0.2% bile salts, and 0.50 ml deactivated steapsin (crude steapsin which had been heated for one hour at 38°C and pH 9,. This treatment destroys a contaminant (lipase) which hydrolyzes the fatty acids on the 2-position of the glycerides.) The reaction mixture was incubated at 37°C for 2 hours with shaking. The reaction was stopped by the addition of 1 ml 1N HCl. The lipids were then taken up in ether, separated on TLC plates, and counted.

RESULTS

Effect of Glycerol Concentration on M. Smegmatis

In the following experiments, 500 mls of Youman's medium in 2.8 l Fernback flasks which contained varying glycerol concentration, were inoculated with 10 mls of log-phase M. smegmatis cells. The flasks were incubated at 26°C for 5 days with shaking. The cells were harvested and washed once with distilled water. The procedures for investigating the different parameters were covered in Materials and Methods.

Table I shows the effect of varying the glycerol concentration on pH, total cell yield, amount of glycerol utilized per gram of cells, percentage of total lipids and percentage of TGs relative to the cells dry weight. As can be seen from the table, growth of the organisms does not markedly affect the pH of the medium. However, the effect of glycerol on cell yield is distinct. The cell yield increased some 20 fold upon addition of 1% glycerol to growth medium. This implies that glycerol acts as both a carbon and energy source for the cells, hence the increase in the yield.

Increasing the concentration of glycerol beyond 1% in the growth medium does not increase cell yield. Both the cell yield and the amount of glycerol utilized remained constant between 1 and 10%. Examination of the yield of total lipids and TGs, however, shows a relative increase in both with increasing glycerol concentrations. This may suggest that the cells have the ability to take a certain limited amount of glycerol from the medium, part of which is metabolized as a source of carbon and energy and the rest is used in making total lipids and TGs. Thus with

Table 1

Effect of Glycerol Concentration on Growth and Lipid Content of M. Smegmatis

% Glycerol in Medium	pH Before Growth	pH After Growth	Total Cell Yield Freeze Dried	Dry Cell Wt / ml*	g Glycerol Utilized/g Cells	% Total Lipids	% TGs
0	7.1	7.5	176.7 mgs			11.7	1.4
	7.1	7.5	161.0			12.5	1.5
1	7.2	7.1	3234	6.190	3.3	17.8	9.3
	7.2	7.1	3247	6.195		17.6	10.0
3	7.1	7.0	3405	6.516		18.4	9.8
	7.1	7.0	3117	6.002	3.6	18.4	9.4
5	7.0	7.0	3795	7.218		20.1	11.1
	7.0	7.0	2670	5.206	3.4	22.3	11.2
10	7.1	7.1	3340	6.378		24.0	13.3
	7.1	7.1	3250	6.187	3.6	24.6	13.0

*Corrected for weight loss (100 C - 24 hours.). Only 2-3% loss.

increasing concentrations of glycerol in the growth medium, the total lipids and the TGs content increase although there was no concomitant increase in cell yield.

Table 2 shows the relation between incubation time and cell yield and TG content, when the cells were grown in different glycerol concentrations. In the two incubation media, the cell yield increases with time significantly, but the percentage of TG does not show a relative increase. The TG content, however, is higher in the medium containing 10% glycerol. This is in agreement with the previous experiments. Furthermore, Table 2 shows that TG synthesis is synchronous with growth contrary to the general belief that organisms first utilize nutrients for synthesis of major products for growth and then use the excess of nutrients for making storage products.

Function of Triglycerides

In the previous experiments evidence was presented that the total lipids and TG content of whole cells increased when the concentration of nutrients (eg. glycerol) in the growth medium was increased. This may suggest that TGs are storage products. If TGs are truly storage products, then depleting the growth medium of the carbon source should lead to their consumption upon prolonged starvation.

In order to verify the last hypothesis cells were incubated with 12.26 uCi of ^{14}C-oleic acid for 30 minutes, collected and washed twice with distilled water. (This procedure was followed in order to label the TGs). Unpublished experiments have shown that 50% of the added fatty acids are incorporated directly into TGs under these conditions.

Table 2

Effect of Glycerol Concentration and Incubation Time on Cell Yield and TG Content

Incubation Time (Days)	2% Glycerol		10% Glycerol	
	mgs Cells/100 mls Medium	% TG / Cell Dry Wt.	mgs Cells/100 mls Medium	% TG / Cell Dry Wt.
2	143	7.9	125	8.9
	128	7.9	133	9.8
3	284	9.1	290	11.0
	336	8.7	238	10.3
4	536	9.2	470	11.7
	547	9.7	501	11.6
5	705	10.6	622	15.2
	659	10.4	658	11.5
6	788	10.6	577	12.6
	775	11.1	745	11.5

The pellet was then resuspended in a carbon free salt medium (KM without glucose). A sample was taken immediately after the cells were suspended in the salt medium (0 time) and the counts were determined in the TGs. Equal samples were taken after 12, 24, and 36 hours and analyzed for TG content. As Figure 2 shows, the radioactivity in the TGs decreased with increased starvation time, but the counts increased simultaneously in the phospholipids then levelled off. This experiment shows that TGs are storage products. When the cells are starved, the TGs are broken down to release free fatty acids (decrease in radioactivity). The fatty acids are then incorporated either in toto in the more important structural phospholipids (increase in radioactivity) or they are used as an energy source being degraded by B-oxidation. The 2-carbon units that are produced could be incorporated in the p-lipids. This point merits further investigation but is beyond the scope of this work.

The cell free extract seems to resemble whole cells in that it synthesizes excess TGs in the presence of excess nutrients. When the crude cell free extract from 3-day old cells was incubated with excess glycerol and increasing concentrations of ^{14}C-palmitic acid, there was a concomitant increase in TG content (Figure 3).

Biosynthesis of Triglycerides

The monoglyceride pathway. As was mentioned earlier, the monoglyceride pathway essentially involves the direct acylation of monoglycerides (MG) by acyl-CoA derivatives. If triglycerides are synthesized via this pathway, then the original MG molecule should be incorporated intact

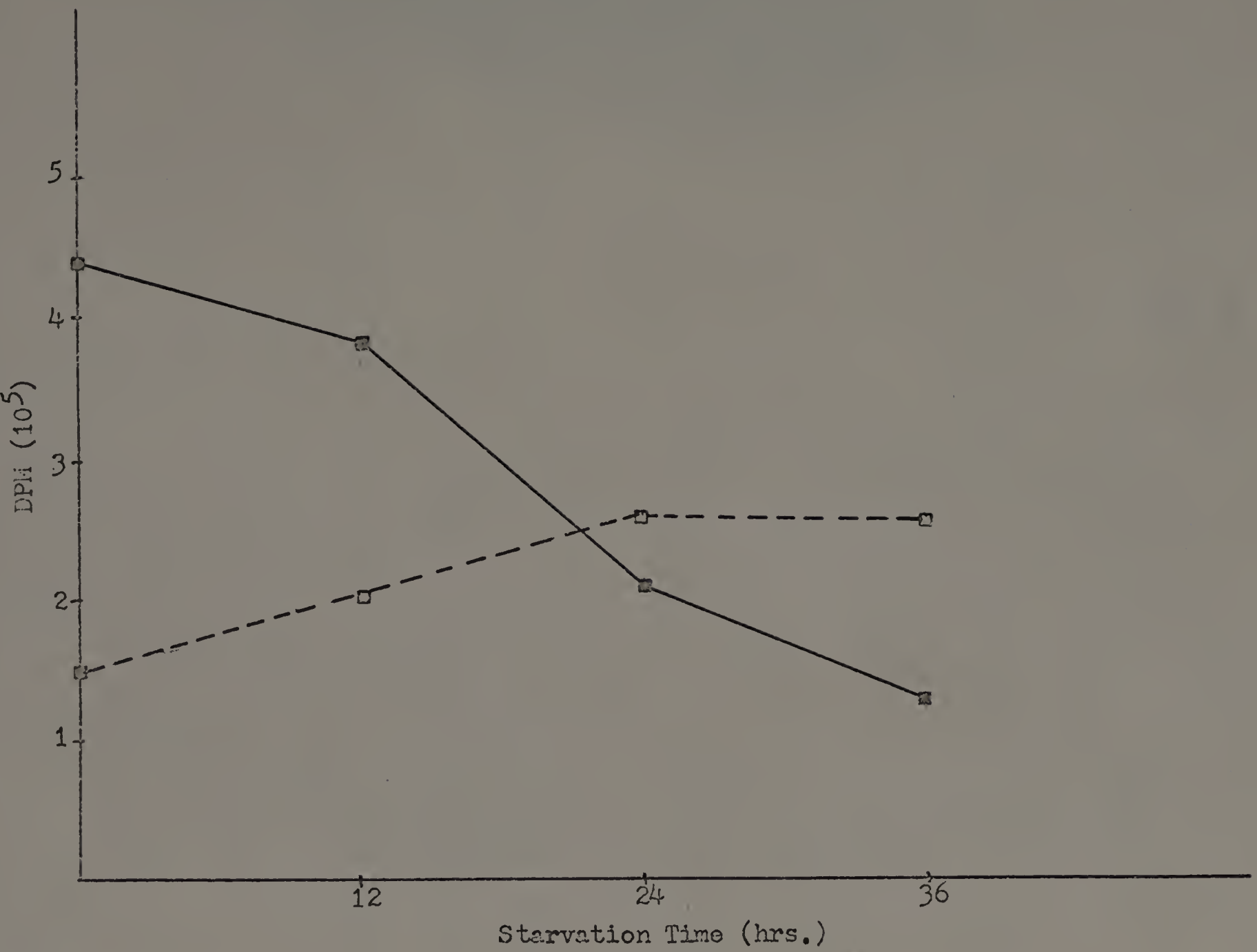


Figure 2

Relation Between Starvation Time and Content of Radioactivity in TGs (-□—□-) and Phospholipids (-□--□-)

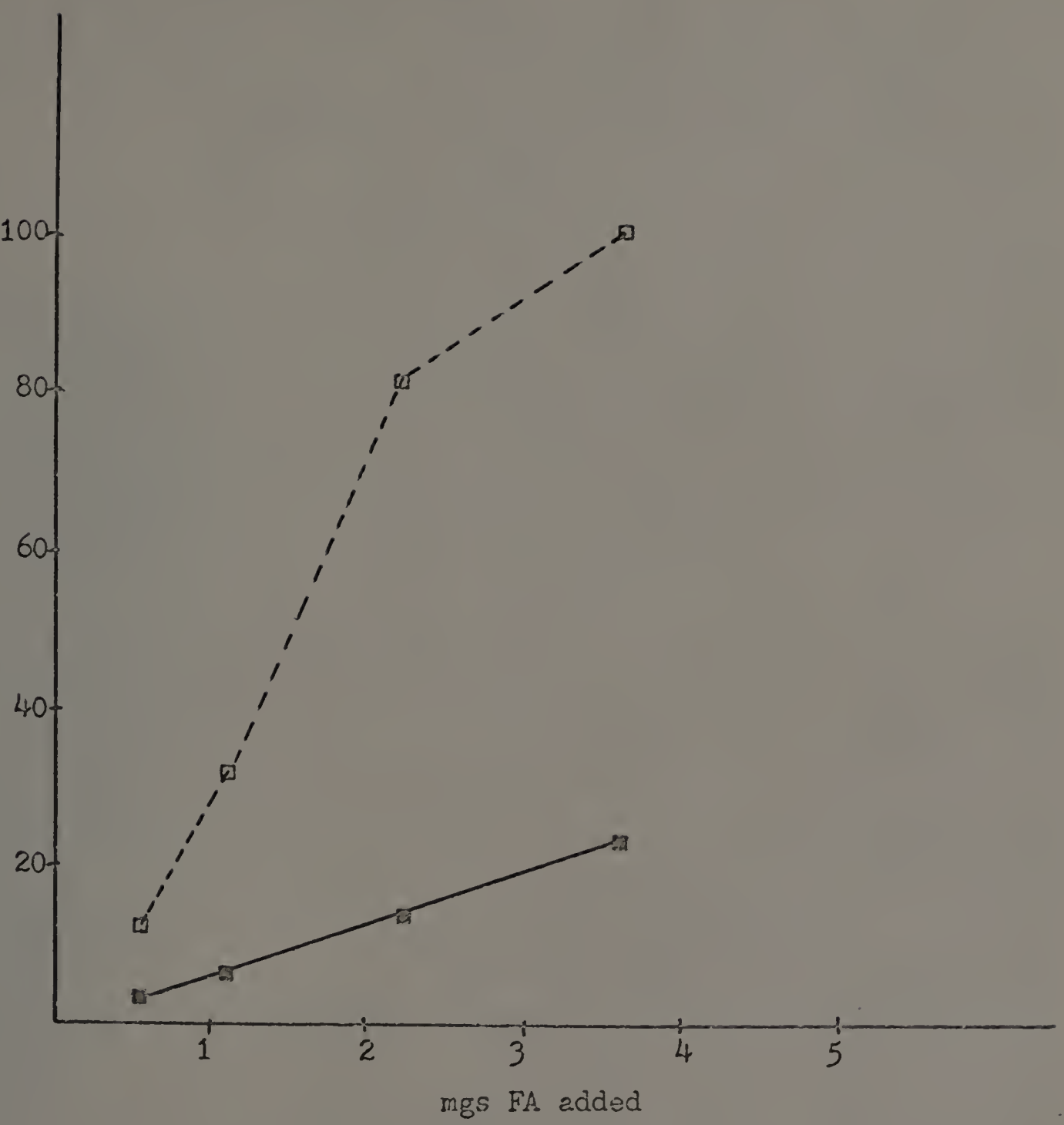


Figure 3

Relation Between Increasing FA Concentration and Incorporation of ^{14}C-palmitic Acid in TGs (-□--□-) and DGs (-■—■-)

into TGs with very little or no change in its basic structure. Thus if a doubly labeled MG, where the ratio of counts of the two radioisotopes is known, is added to a cell free system, and the same ratio of counts is recovered in the TGs, then this is a clear indication that the MG molecule is incorporated intact into the TGs by being directly acylated without disturbing the original ratio. Furthermore, if the MGs are intermediates in either a major or a minor pathway, addition of MGs to a cell free system supplemented with the necessary cofactors for TG synthesis should enhance such a synthesis.

To verify whether the MG pathway operates to any extent in M. smegmatis, and to see if MGs are intermediates in any pathway, the following experiments were done:

To 0.50 ml cell free extract from young cells, 1 umole palmityl-CoA, 1 umole chemically synthesized doubly labeled MG [the MG had 74,280 cpm of ^{3}H-glycerol and 211, 540 cpm ^{14}C-palmitic acid ($^{3}H/^{14}C$ ratio of 0.35)], 25 um NaF, 10 um $MgCl_2$ and 0.1 ml of 10% Tween-80 were added. Tween-80 was added because it is assumed to inhibit the a -GP pathway (59) thus making the MG pathway more prominant in the synthesis if such a pathway exists. Table 3 shows the ratio of counts in all the compounds isolated. The ratio of $^{3}H/^{14}C$ in the TGs is nearly doubled implying that there is significant hydrolysis of the FA of the MG. Furthermore, high counts of ^{3}H in the FA fraction suggest that probably after hydrolysis of the MG, the glycerol fraction of the molecule was metabolized to make more fatty acids.

In another similar experiment and in an attempt to minimize hydrolysis, the crude CFE was fractionated by centrifugation at 40,000 x g for one hour. Both the supernatant fraction and the pellet were used in this experiment. The conditions of the reaction were essentially the same as the previous one except that the doubly labeled MG was isolated from cells that had been incubated with ^{3}H-glycerol and ^{14}C-palmitic acid. The total counts added were ^{3}H-3,430 and ^{14}C-10,322; and the ratio of ^{3}H/^{14}C in the MG was 0.31. Table 4 shows that the ratio of counts in the TGs and the FAs in both the pellet and supernatant fractions are altered.

To determine whether MG acts as an intermediate, crude CFE and the pellet fraction were separately incubated with ATP, CoA, a-GP, Mg^{++}, Ni^{++} and MGs. As a control, the same reaction mixtures were incubated with diolein. Table 5 shows the results. In the presence of diolein, the incorporation of ^{14}C-oleic acid in the crude cell free extract was high. Substituting monolein for diolein, however, did not produce the same effect. No noticeable effect is seen when monolein is incubated with and without Tween-20 in the reaction mixture. This suggests that monoglycerides are not intermediates in either the monoglyceride pathway or the a-G-P pathway. The role of diolein will be discussed in another part of this thesis.

The a-Glycerophosphate Pathway. Previous experiments showed that the monoglyceride pathway does not contribute to TG synthesis in any measurable way. This implies that TGs are synthesized through another pathway. Since M. smegmatis grows well on glycerol and since it was

Table 3

Utilization of (^{3}H, ^{14}C)-monopalmitin in Crude Cell-Free Extracts

Compound	^{3}H cpm	^{14}C cpm	Ratio $^{3}H/^{14}C$
TG	532	865	0.61
FA	22170	60310	0.39
DG	2462	5290	0.46
MG	1101	2701	0.40
CL	1055	1594	0.66
PA	746	870	0.46
PE	639	646	0.98
PIM	521	324	1.60

The incubation mixture contained 1 umole doubly labeled monopalmitin (contains ^{3}H, 74,280 cpm and ^{14}C, 211,540 cpm with a ratio of 0.35), 0.5 ml 0.05 M phosphate buffer, 10 umoles $MgCl_2$, 25 umoles NaF, 1 umole palmityl-CoA, 0.1 ml 10% Tween-80 solution and 0.5 ml cell-free extract. Total volume was 1.8 ml. Incubation was for 1 hr. Reaction was stopped by the addition of 3 ml C:M (2:1).

Table 4

Utilization of (^{3}H, ^{14}C)-monopalmitin by Pellet and Supernatant Fractions

Compound	Pellet Fraction			Supernatant Fraction		
	^{3}H (cpm)	^{14}C (cpm)	Ratio	^{3}H (cpm)	^{14}C (cpm)	Ratio
TG	90	78	1.15	85	57	1.49
FA	510	1059	0.48	1003	2049	0.48
DG	101	114	0.88	110	120	0.90
MG	1947	3780	0.51	1507	3555	0.42
O	242	255	0.94	232	219	1.05

The incubation mixture contained 2 umoles doubly labeled monopalmitin (containing 3-H, 3430 cpm and 14-C 10,332 cpm with a ratio of 0.31), 1 ml of either pellet or supernatant, 0.60 ml buffer, 5 umoles oleic acid, 5 umoles ATP, 0.2 umoles CoA and 20 umoles Mg^{++} in a total volume of 1.5 ml. The reaction was stopped by adding 3 mls C:M (2:1) after 1 hr. Incubation at 38°C.

Table 5

The Effect of Monolein and Diolein on Triglyceride Synthesis in Crude CFE and Pellet

	Cell Free Extract									Pellet			
	Diolein				Monolein					Diolein		Monolein	
Compound	All*	None	Minus GP	Minus Mg^{++}	All	None	All + T-20	Minus GP	Minus MG^{++}	All	All + T-20	All	All + T-20
TG	180,176**	3,664	118,943	159,965	5,184	1.872	3,500	4,546	4,088	37,870	15,732	4,856	4,510
DG	6,031	3,305	4,561	3,959	5,270	3,003	4,044	5,543	4,605	6,168	7,816	3,724	5,600
MG	1,211	1,177	1,881	2,506	1,697	1,015	1,050	1,409	1,530	1,951	2,836	1,776	2,017
O	3,584	1,365	3,903	4,623	2,093	1,138	1,469	1,703	1,968	3,856	6,444	2,203	2,092

*The complete mixture contained 10 umoles ATP, 7 umoles a-GP, 0.25 umoles CoA, 10 umoles $MgCl_2$, 10 umoles $NiCl_2$ and 0.48 uCi 14-C oleic acid suspended in 0.3 ml of 5% BSA. The reaction vessels were incubated at 38°C for 1 hr. The reaction was stopped by adding 3 mls C:M (2:1) containing 0.025 ml 2 N HCL. 5 umoles of either monolein or diolein were included in the incubation mixture.

**CPM

shown that glycerol is necessary for TG synthesis, it was chosen to be used as a marker to trace synthesis.

Young cells were collected by centrifugation and resuspended in 50 mls of basal medium. To the suspension 5 uCi of ^{14}C-glycerol (Sp. Act. 20 mCi/mM) were added and the suspension was incubated at room temperature for 5 minutes with constant stirring. The cells were collected my millipore filtration, washed, and the lipids extracted as was described in Materials and Methods.

Table 6 shows the results of pulse-labeling the cells with ^{14}C-glycerol. Of significance are the counts in a-GP and PA suggesting that TGs are synthesized through the a-GP pathway. The low counts in a-GP may be explained by the fact that this compound is the first compound in the pathway derived from glycerol and has a high rate of turnover. It is suspected that the moment a-GP is synthesized, it is acylated to produce lyso-PA. Lyso-PA was not isolated because no system for separating it from other phospholipids was found. The fact that counts were detected in the PA fraction shows that it is an intermediate in the pathway. Although it has been shown that PA has a very high rate of turnover in mammalian systems, it seems that its turnover rate in M. smegmatis is not as high as that of a-GP or lyso-PA.

If a-GP, lyso-PA and PA are intermediates in the synthesis of TGs with a relatively high rate of turnover, then pulsing whole cells with a radioisotope for increasing time intervals should show a concomitant decrease in the counts of these compounds. To check this hypothesis, young cells were incubated with 4 uCi of ^{3}H-glycerol (Sp. Act. 420 mCi/mM).

Table 6

Pulse-Labeling Whole Cells With
^{14}C-glycerol for 5 Minutes

Compound	Counts/Minute
TG	7,500
DG	2,000
FA	1,160
MG	4,004
aGP	2,086
CL	4,700
PA	26,960
PE	60,000
PIM	22,560

Equal samples were taken after 1, 2, 10 and 30 minutes, treated as was described previously and the isolated compounds counted. The results in Table 7 show that intermediates with a high rate of turnover showed either decreasing counts with increasing time of pulse or low counts throughout the time of pulse. End products, like CL, PIM, and TGs, on the other hand, showed an increase in incorporation of glycerol and had the highest counts after the longest time of exposure to the isotope. The fluctuating, but high counts, in PE could be due to the fact that PE itself turns over and acts as an intermediate in a very strict sense.

Pulse-Chase with Glycerol

In order to determine which lipid compounds have the highest rate of turnover and be able to establish which ones are intermediates and which are end-products, a pulse-chase experiment, the details of which were previously described, was performed. Table 8 shows the results. It is evident that PA has the highest rate of turnover followed by PE. This is in agreement with the original hypothesis that PA is an intermediate and that PE, although not an intermediate per se, shows some turn over. End products like cardiolipin and PIM show little turnover thus abiding to their role as basic structural compounds and building blocks of the cells. TGs are also stable and show little turnover indicative of a role as storage compounds.

Pulse-Labeling Whole Cells with Two Isotopes

Table 9 shows the results of pulse-labeling whole cells with 2 isotopes simultaneously, and Figure 4 shows the incorporation of ^{14}C-palmitic acid in TGs and DGs. The noticeable low counts in most compounds

Table 7

Pulse-Labeling Whole Cells for Different Time Intervals with ^{3}H-glycerol

Compound	1 Min.	2 Min.	10 Min.	30 Min.
TG	580	477	667	2017
DG	560	431	460	565
FA	520	552	588	659
aGP	568	428	348	288
CL	525	510	756	2021
PA	575	542	486	565
PE	4191	2928	3334	3187
PIM	680	526	648	899

Table 8

Pulse-Chase with Glycerol

Time	TG	DG	MG	CL	PA	PE	PIM
Before suspension in 1% glycerol	1,035,890*	71,681	12,400	109,015	28,500	52,654	70,766
After suspension in 1% glycerol	856,109	53,345	3,700	111,107	26,333	47,890	54,109
10 min. chase	896,360	48,433	2,678	76,584	31,963	41,236	81,236
30 min. chase	778,311	53,800	4,117	82,270	29,015	42,800	61,166
60 min. chase	695,818	38,433	4,000	51,666	13,626	28,533	56,466

*DPM

Table 9

Pulse-Labeling with ^{3}H-glycerol and ^{14}C-palmitic Acid

	1 Min.		5 Min.		10 Min.		30 Min.		45 Min.		60 Min.	
Compound	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
TG	606	1,109	983	1,879	1,747	3,311	2,425	6,411	4,649	9,120	9,346	23,963
DG	692	1,608	961	1,982	1,007	2,141	1,052	2,304	1,418	3,693	2,437	6,269
MG	438	1,089	325	692	378	713	672	941	315	657	531	1,027
CL	182	167	149	120	178	167	165	167	211	288	375	614
PA	412	481	298	318	321	391	290	356	377	593	790	1,019
PE	486	193	215	141	266	150	685	270	698	374	812	769
PIM	182	124	165	124	158	107	231	210	323	369	475	546

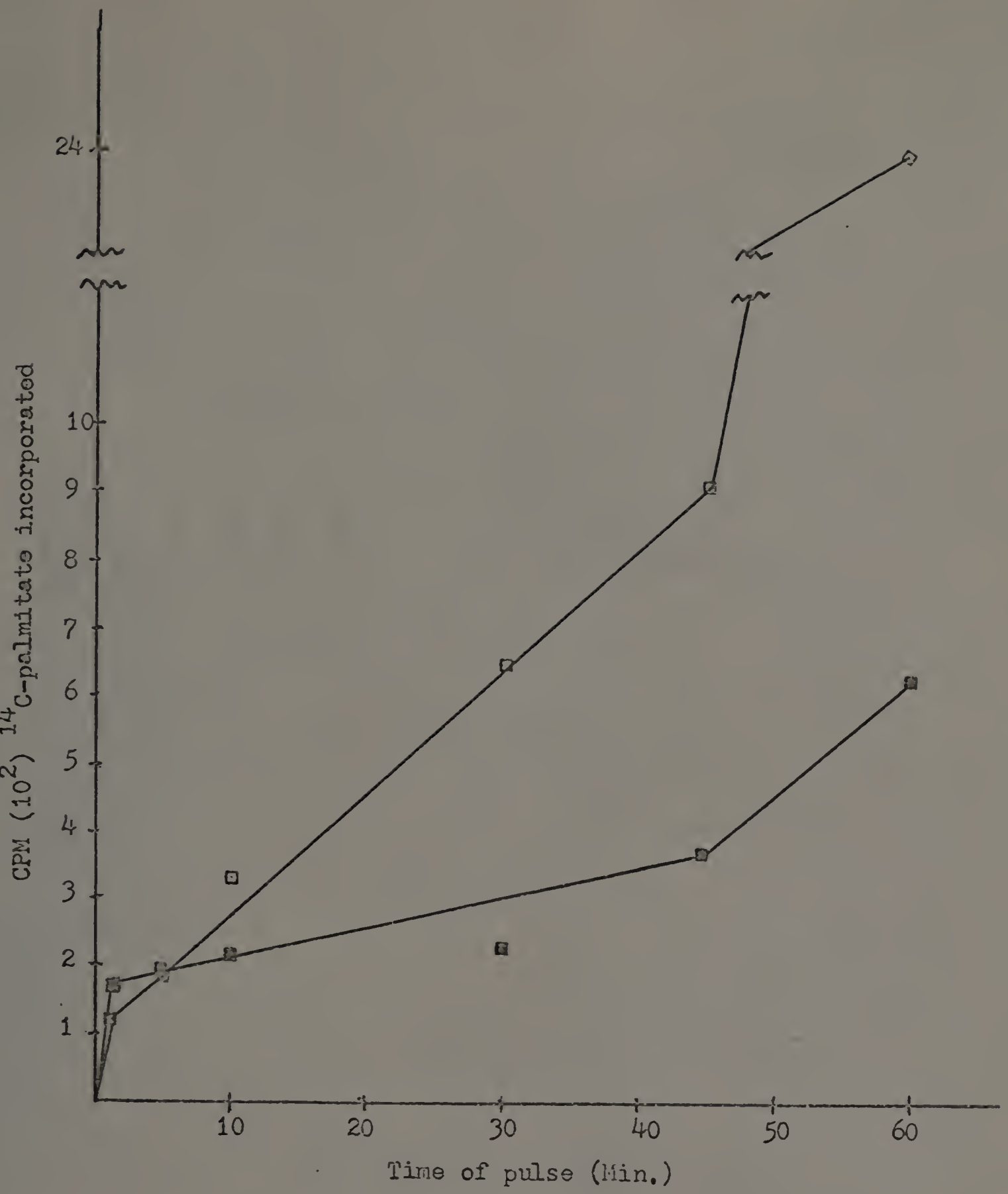


Figure 4

Incorporation of ^{14}C-palmitic Acid in the TGs (-□—□-) and DGs (■——■)

Whole cells were pulse-labeled with ^{3}H-glycerol and ^{14}C-palmitic for different times. Conditions of the experiment are covered in the text.

is due to dilution of both isotopes in the medium. Contrary to previous experiments, incorporation of the isotopes in the end products is not high even after long exposure to the isotopes. However, glycerol is incorporated to a greater extent than the fatty acids in the phospholipids. The different degree of incorporation of glycerol and fatty acids could be related to differences in their rate of turnover. The fatty acids are physically more accessible to the catalytic activity of enzymes such as lipases and transacylases and could be easily interchanged with non-labeled fatty acids. Glycerol, on the other hand, is liable to turnover much less than the fatty acids since it acts as a backbone for the synthesis of the p-lipids.

Experiments with Cell-Free Extract

From previous experiments both in cell-free extracts and whole cells, evidence has been presented that the -GP pathway, and not the MG pathway, is the pathway involved in TG synthesis in M. smegmatis.

GP and PA were shown to be intermediates in that pathway as evidenced by the pulse labeling and chase experiments.

In order to study the different reactions that lead to TGs and to determine what regulatory mechanisms are involved in such a synthesis, it was important to reconstruct an in vitro system that could resemble the whole cells. Theoretically, the best system which would resemble whole cells is the cell-free extract since it contains the same enzymes that the cell contains, provided that the cofactors required for optimal catalysis are known and are added to the CFE. However, when the cell is disrupted, it loses its integrity and organization. Thus instead of

having certain reactions "compartmentalized" in certain regions of the cell where they would be more easily controllable, a chaotic situation is generated. The result is a loss of control of certain enzymes and a disturbance in related reactions. This situation occurred in the in vitro system of M. smegmatis. ATPase was found to be very active and destroyed the endogenous as well as the exogenous ATP, particularly in CFE prepared from young cells. It was found that 0.27 umoles ATP are hydrolyzed/20 minutes/mg protein in CFE from 3-day old cells; 0.14-0.25 umoles ATP are hydrolyzed/20 minutes/mg protein in CFE from 4-day old cells; 0.03 umoles ATP are hydrolyzed/20 minutes/mg protein from 7-day old cell-free extract. To inhibit ATPase, fluoride and nickel ions were tried. Fluoride ions inhibited ATPase up to 52% whereas nickel ions exerted a 100% inhibitory effect of ATPase at a concentration of 10 umoles or higher. Consequently, in most experiments conducted with the cell-free extract, 10 umoles of $NiCl_2$ were routinely added to the incubation mixture.

Several attempts were made to construct a cell-free system that resembles whole cells. The best working model was found to require ATP, CoA, aGP, magnesium ions, nickel ions and diolein. Table 10 shows the degree of incorporation of ^{14}C-oleic acid in such a system. In the presence of all the factors mentioned above, 42% of the added oleic acid was incorporated into the TGs, whereas there was less than 1% incorporation in the absence of these factors. Omitting aGP from the reaction mixture resulted in 26% incorporation of the labeled fatty acid, whereas eliminating Mg^{++} ions did not have a great effect. Addition of Tween-20

Table 10

Incorporation of ^{14}C-oleic Acid in Glycerides

in the Working Cell-Free Extract Model

Compound	All Factors	No Factors	All + Tween-20	All (-) a-GP	All (-) Mg^{++}
TG	40.8*	0.83	21.9	27.2	35.7
DG	1.35	0.72	1.21	0.99	0.85
MG	0.32	0.28	0.47	0.45	0.59
O	1.11	0.32	2.13	1.13	1.30

The control tube contained 1 ml CFE, 10 umoles ATP, 0.25 umoles CoA, 7 umoles a-GP, 10 umoles Mg^{++}, 10 umoles Ni^{++}, 96 nmoles ^{14}C-oleic acid (0.48 uCi) and 5 umoles diolein in a total volume of 1.8 ml. The reaction tubes were incubated at 37°C for 1 hr. and the reaction was stopped by adding 3 mls C:M (2:1) containing 0.025 ml 2 N HCl.

* nmoles oleate incorporated.

to the incubation mixture allowed only 21% of the added ^{14}C-fatty acid to be incorporated into the TGs. Tween-20 inhibits the aGP pathway at the PA level. The fact that there was incorporation of the isotope in the TGs suggests that Tween-20 partially inhibits the pathway. To further examine the effect of detergents on TG synthesis and hence deduce the role of PA in such a synthesis, several detergents were separately included in the incubation mixture, and the results are shown in Table 11. These experiments support the hypothesis that PA is an intermediate in the pathway that leads to TGs. Inhibiting the pathway at the PA level, reduces incorporation of the labeled fatty acid tremendously.

The role of diolein that was included in the original reaction mixture in TG synthesis was investigated further. In one experiment, incorporation of ^{14}C-oleic acid was studied in the presence and absence of diolein, and the results are shown in Table 12. Incorporation of the isotope in the incubation mixture that did not contain diolein was higher than the incorporation in the mixture containing diolein. This implies that diolein (which is mainly 1,3-DG) is not a direct precursor of TGs, possibly because it does not have the correct configuration necessary for direct acylation. This experiment led to investigating the identity of the direct precursor of TGs. Other possible precursors were included in the reaction mixture where all the factors necessary for TG synthesis were included. However, instead of using ^{14}C-oleic acid as a marker, ^{14}C-24-CoA (chemically synthesized) was used because it was shown from previous work (Walker, et. al. (69)) that C-24 is

Table 11

Effect of Different Detergents on Triglyceride Synthesis

		Cutscum			Sodium Lauryl SO_4			Zephiran		
Compound	Control	0.1%	0.5%	1.0%	0.15 um	0.75 um	1.5 um	0.4 um	2 um	12 um
TG	82,918*	24,333	10,278	2,828	18,563	419	399	51,475	999	314
DG	2,370	9,180	3,750	5,925	4,976	4,411	3,941	2,365	2,567	4,002
MG	211	2,496	1,262	1,883	1,333	603	489	229	448	374
O	7,908	3,937	3,418	1,266	4,610	318	582	4,281	3,225	124

The reaction mixtures were essentially the same as those that were mentioned in Table 10 except that diolein was not included.

*CPM oleate incorporated.

Table 12

Effect of Diolein on the Incorporation of ^{14}C-oleic Acid

in Cell-Free Extracts

Compound	All Factors + Diolein	All Factors (-) Diolein
TG	316,655*	384,596
DG	28,718	15,804
MG	11,316	6,464
O	16,016	23,687

Conditions of this experiment were the same as those mentioned in Table 10.

*DPM

preferentially added to the third carbon of glycerol. Table 13 shows that incorporation of the labeled long-chain fatty acid was highest in the reaction mixture containing 1,2-DG. Of the 360 nmoles of ^{14}C-24-CoA added, 37 nmoles were incorporated into the TGs when 1,2-DG was included in the reaction mixture, whereas the first control which had no acceptor allowed 14 nmoles incorporation. Addition of PA did not affect incorporation significantly, possibly because other additional factors are necessary for the involvement of PA. Table 14 shows the results when the same tracer (^{14}C-24-CoA) was used in the presence of other factors like CTP and ACP. Incorporation of the isotope was high in the presence of 1,2-DG, and a little higher when PE, crude ACP and CTP were included in the incubation mixture. Neither PA alone, nor PA plus CTP affected synthesis significantly when they were included in the incubation mixture.

In a more varied experiment, several compounds were tested to see if any of them could be the direct precursor of TGs. Table 15 shows that GSH, a-GP, PE, ACP and glycerol are important factors in the synthesis. Because of the increase in incorporation of the labeled fatty acid in the presence of GSH, DTT was included in the buffer system. The role of a-GP, PE, and ACP will be discussed in another part of this thesis.

Role of Added Factors

As was mentioned, the best working cell-free model required ATP, CoA, aGP, Mg^{++} and Ni^{++} for best incorporation of ^{14}C-oleic acid in the TGs. The importance of each factor was determined in this experiment. The control tube contained all the factors; the amount of labeled fatty acid incorporated in the TGs was taken to be 100%. Incorporation in other

Table 13

Effect of Different Compounds on Triglyceride Synthesis

Compound	Control + Ni Ions	Control + 10 um ATP + Ni	Control + 5 um PA	Control + 5 um PE	Control + 5 um 1,2-DO**	Control + 5 um 1,3-DO***
TG	380*	643	631	725	1,023	926
FA	4,327	4,549	4,080	4,454	4,495	4,567
DG	217	189	76	95	89	98
MG	70	89	247	55	44	58
C	551	520	298	860	299	307

The control mixture contained 360 nmoles ^{14}C-24-CoA, 10 umoles Ni, 1 ml CFE, and 0.3 ml 5% BSA.

* CPM ^{14}C-24-CoA incorporated
** 1.2/1,3-DO ratio is ca. 50:50
*** 1,3/1,2-DO ratio is ca. 85:15

Table 14

Effect of CTP and ACP on TG Synthesis in the Presence of Other Factors

Compound	5 um of PE	5 um PE +5 um CTP	20 mgs ACP +5 um PE +5 um CTP	1,2-DG	1,2-DG + CTP	PA + CTP	PA
TG	356*	405	670	624	580	546	465
FA	4,648	2,066	2,094	2,056	4,893	3,458	4,499
DG	112	48	94	118	134	91	60
MG	45	40	51	42	51	70	564
O	688	595	640	125	148	550	624

Conditions of this experiment were similar to those shown in Table 13.

*CPM ^{14}C-24 incorporated

Table 15

Effect of Different Compounds on TG Synthesis

Compound	Control (1) + a-GP	Control (1) No Additions	Control-2 + GSH	Control-2 No a-GP	Control-2 + 15 um Glycerol	Control-2 + 1 mg/ml Glycerol Kinase	Control-2 + 5 um Lyso-PA	Control-2 + 5 um PA
TG	76,279*	1,339	106,250	92,890	84,706	69,626	53,134	19,785
DG	4,079	1,721	5,420	6,786	2,110	----	8,478	2,154
MG	2,519	1,488	923	3,467	1,313	3,045	1,142	3,959
O	3,526	960	4,756	7,220	3,562	3,175	14,502	2,261

Conditions of this experiment are essentially the same as those shown in Table 13.

*CPM oleic acid incorporated

Table 15 Continued

Effect of Different Compounds on TG Synthesis

Compound	Control-2 + Lyso PE	Control-2 + PE	Control-2 + CTP	Control-2 + 10 mgs ACP	Control-2 + 50 mgs ACP	Control-2 + ACP from A. Globiformis	Control-2 + 1,3-DG	Control-2 + 1,2-DG
TG	16,552	144,500	80,760	98,646	99,340	98,043	53,344	63,168
DG	----	4,415	2,986	----	8,197	3,392	----	----
MG	2,040	1,340	1,430	1,717	1,233	1,073	2,205	1,657
O	11,953	12,999	3,955	4,644	8,570	5,871	2,472	3,577

tubes where a factor was depleted at a time was measured in relation to this control. Table 16 shows the results. Depleting CoA or ATP from the system drops incorporation to 5% and 13% respectively. This is expected since both factors are directly involved in activating the fatty acid before it is incorporated into the TGs. It is evident, too, that the levels of these factors in the cell-free system are low and hence they have to be supplied to the incubation mixture. In contrast to these two factors, a-GP inhibits the system as evidenced by the drop in incorporation when it was included in the reaction mixture. The role that a-GP plays in TG synthesis is conflicting: at certain times it induces TG synthesis (Table 10), at other times (Table 16) it inhibits it. a-GP was found to play different roles depending on the age of the cells from which the cell-free extract was prepared. This point will be discussed in detail in a latter part of this thesis.

Because of the high incorporation of the labeled fatty acid in TGs, it was suspected that what is detected is only the last step in the pathway where the fatty acid is acylated to the third carbon of a preformed intermediate. Two approaches were followed to examine this point: verification of de novo synthesis and direct analysis of the structure of the TGs synthesized in vitro.

De Novo Synthesis

Table 17 shows the results of two parallel experiments that were identical in all respects except in the nature of the tracer used. One experiment contained ^{14}C-palmitic acid, and the other contained ^{14}C-oleic acid as markers. Although the amount of labeled oleic acid incorporated

Table 16

Relative Incorporation of ^{14}C-oleic Acid in Different Systems

System	DPM in TGs	Relative Incorporation
Complete *	45,263	100%
Complete (-) ATP	6,275	13%
Complete (-) CoA	2,272	5%
Complete (-) a-GP	74,496	164%
Complete (-) Mg^{++}	28,182	62%
Complete (-) Ni^{++}	39,636	87%

*The complete system contained 1 ml CFE, 15 umoles ATP, 0.50 umoles CoA, 14 umoles a-GP, 10 umoles Ni, 15 umoles Mg and 0.31 uCi 14-c-oleic acid in 0.3 ml of 5% BSA. Cells were 7 days old, and the CFE had a protein concentration of 14 mgs/ml.

Table 17

Incorporation of ^{14}C-oleic and ^{14}C-palmitic Acid in Different Compounds in Relation to Time of Incubation

Compound		5 Min.	15 Min.	30 Min.	60 Min.	90 Min.	120 Min.
TG	16:0	4.2*	13.7	15.0	16.4	12.3	21.8
	18:1	5.7	17.7	21.7	20.1	31.0	27.4
	18:1/16:0	1.35/1	1.29/1	1.44/1	1.22/1	2.52/1	1.26/1
DG	16:0	1.1	1.0	1.2	1.2	----	1.9
	18:1	0.3	0.3	0.4	0.5	0.7	0.4
MG	16:0	0.23	0.31	0.18	0.17	0.14	0.15
	18:1	0	0	0	0	0	0
O	16:0	1.5	3.1	3.3	3.0	3.1	2.0
	18:1	0.6	1.4	1.6	1.5	1.2	0.6

Conditions of this experiment are the same as those that appear in Table 10.

*nmoles of the labeled fatty acids incorporated

was slightly higher than that of palmitic acid, the ratio of 18:1/16:0 in the TGs remained fairly constant with increasing time. If the incorporation was limited to the last step of the pathway, the ratio of incorporation of the two isotopes should vary. One of the two fatty acids would be acylated to the third carbon of the intermediate and consume it in a short period of time to complete the pathway. In this event, the ratio will not remain constant with the increase in the time of incubation.

More significant is the degree of incorporation of each isotope in the DGs and MGs. If acylation is restricted to the third carbon, no counts should be detected in the DGs, MGs or phospholipids (origin). Furthermore, this experiment shows that acylation of the glycerol molecule (or a derivative of it) occurs on the second carbon first. From previous work (Walker et. al.) it was established that palmitic acid is preferentially esterified to the second carbon of glycerol. In this experiment, the isolated MGs were acylated with palmitic acid only regardless of the time of incubation.

To further elucidate the existence of de novo TG synthesis, cell-free extracts from young, starved and old cells were incubated separately with either ^{14}C-glycerol or ^{14}C-oleic acid of equal specific activity. Table 18 shows the degree of incorporation of each isotope. The evident difference in incorporation of each isotope could be due to either the non-utilization of glycerol in de novo synthesis or to a lack of some factor(s) in the system where ^{14}C-glycerol was used. Free fatty acids (necessary for TG synthesis) are found in trace amounts in the cell and

Table 18

Incorporation of ^{14}C-glycerol and ^{14}C-oleic Acid in the TGs of CFE From Young, Starved and Old Cells

Compound	DPM FA Incorporated	DPM Glycerol Incorporated
TG from young cells	122,876	249
TG from old cells	115,500	239
TG from starved cells	84,004	176

Conditions of this experiment were the same as in Table 10. The two labeled products that were included in the cell-free extracts were of equivalent specific activity.

hence can act as limiting factors causing the low incorporation of ^{14}C-glycerol in TGs. If this is true, then addition of fatty acids to the system should lead to higher incorporation of ^{14}C-glycerol in the TGs. Two experiments were done to verify this point. In the first, cell-free extract from young cells was incubated with ^{3}H-glycerol, ^{14}C-palmitic acid and buffer only (Table 19). In the second experiment, however, all the necessary cofactors for TG synthesis were included in the incubation mixture along witn ^{3}H-glycerol and ^{14}C-oleic acid (Table 20). The low incorporation of either isotope in the first experiment is due to the lack of the factors necessary for TG synthesis. Once these factors are added, incorporation of ^{3}H-glycerol and ^{14}C-oleic into TGs was extremely high (Table 20).

Structural Analysis of TGs

From the previous experiments, evidence of de novo TG synthesis was presented. The degree of de novo synthesis can be determined by examining the distribution of fatty acids on the synthesized TGs. Cell-free extracts from 3-day old cells was incubated with either ^{14}C-oleic acid (1.8 uCi) or ^{14}C-palmitic acid (3 uCi) with all the other factors. The synthesized TGs, labeled with ^{14}C-fatty acids, were isolated and treated with lipase as previously described. Table 21 shows the results. When the TGs labeled with ^{14}C-oleic acid are hydrolyzed, the bulk of the counts were recovered in the fatty acid fraction. This suggests that the oleic acid is mainly acylated to either position 1 or 3 of glycerol. Some counts, however, were also isolated in the MG fraction suggesting that oleic acid is also acylated to position 2. In the case of palmitic

Table 19

Incorporation of ^{3}H-glycerol and ^{14}C-palmitic Acid in Different Compounds

	1 Min.		5 Min.		10 Min.		30 Min.		60 Min.	
Compound	^{3}H CPM	^{14}C CPM	^{3}H CPM	^{14}C CPM	^{3}H CPM	^{14}C CPM	^{3}H CPM	^{14}C CPM	^{3}H CPM	^{14}C CPM
TG	1,072	2,114	970	1,802	1,006	1,706	1,304	2,028	1,102	2,364
DG	3,886	11,198	5,622	13,316	4,017	8,942	4,930	11,660	4,538	11,968
MG	914	2,576	1,516	3,582	910	2,232	1,311	2,592	1,016	1,120
CL	216	210	309	296	286	324	258	260	250	322
PA	1,924	1.982	4,359	3,210	4,215	3,148	3,089	2,804	2,326	3,430
PE	6,052	248	5,420	242	4,820	360	5,134	256	4,000	244
PIM	419	80	544	102	281	128	352	142	286	72

Cell-free extracts were incubated with either ^{3}H-glycerol or ^{14}C-palmitic acid. No additional factors were included in the incubation mixture. Other conditions of the experiment were the same as in previous ones.

Table 20

Incorporation of ^{3}H-glycerol and ^{14}C-oleic Acid

into the Different Glycerides

Compound	^{3}H	^{14}C	Ratio $^{3}H/^{14}C$
TG	1,355,190*	407,922	3.3
DG	37,282	9,884	3.7
MG	6,958	2,378	2.9

Equimolar concentrations of ^{3}H-glycerol (1 uCi) and 14-C oleic acid were included with all the factors mentioned in Table 10.

*DPM

Table 21

Lipase Hydrolysis of TGs Labeled with Either
^{14}C-oleic or ^{14}C-palmitic Acid

Compound	^{14}C-oleic	^{14}C-palmitic
TG	1,641*	236,164
FA	134,168	314,858
DG	7,324	70,183
MG	3,430	31,244

Cell-free extracts were incubated with labeled oleic or palmitic acids, and all the factors necessary for TG synthesis. The labeled TGs were isolated and treated with lipase.

*CPM

acid, the high counts recovered in the TGs imply that there was incomplete hydrolysis and no definite conclusions could be made. Nonetheless, the fact that there were high counts in the MG fraction also shows that palmitic acid is added to the second carbon of glycerol. Although palmitic and oleic acids were proven to be acylated at specific positions of the TG (position 2 or 1 respectively) it seems that the same degree of specificity does not apply to the cell-free system. These acids were isolated from positions 1, 2 and 3.

These results show that de novo TG synthesis occurs in cell-free extracts to a small extent. Most of the labeled fatty acid is acylated to the third carbon of glycerol.

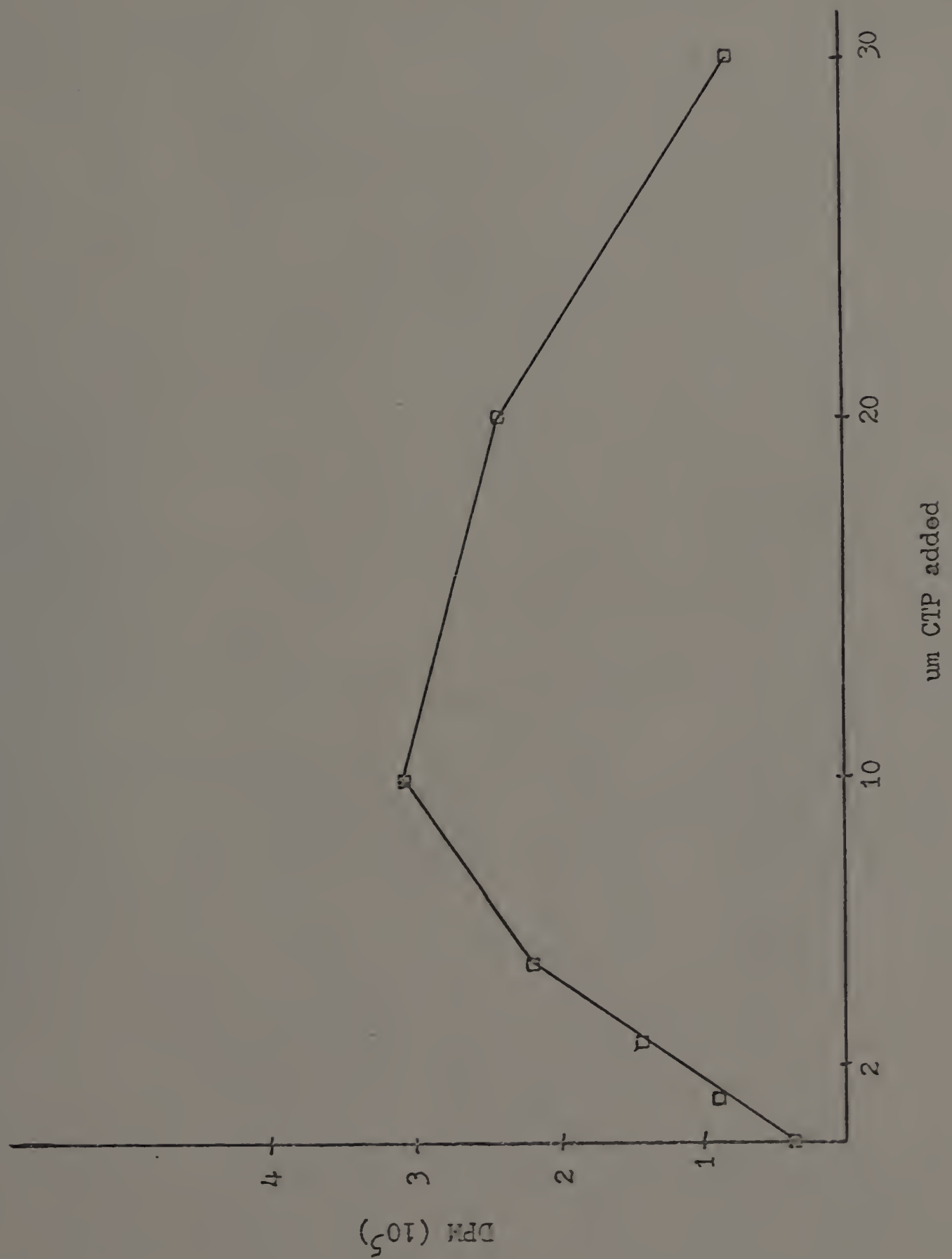
Evidence of CDP-DG as an Intermediate

The conflicting results that were obtained regarding the role of PA in TG synthesis led to the suspicion that another intermediate, probably between PA and DG, (whose synthesis is regulated by either or both) might be responsible for such results. Several authors have isolated and characterized CDP-DG in many microorganisms (E. coli, Micrococcus cerificans, Saccharomyces cerevisiae) but failed to establish the role it plays in lipid synthesis. CDP-DG might exist in M. smegmatis and might be involved in TG synthesis at some level. The first evidence of its existence was obtained when the effects of CTP on TG synthesis were studied. When the cell-free extracts were incubated with varying concentrations of CTP, TG synthesis increased up to a point then dropped sharply (Figure 5). Furthermore, when CTP was included either alone or with other compounds (such as ACP, 1,2-DG) in the cell-free system, TG

Figure 5

Effect of CTP concentration on TG Synthesis

The reaction mixtures contained 1 ml CFE (prepared from 3-day old cells and had a protein conc. of 11.2 mgs/ml), 10 um ATP, 0.25 um CoA, 10 um Mg^{++}, 10 um Ni^{++}, 7 um a-GP, 0.28 uCi ^{14}C-oleic acid suspended in 0.3 ml 5% BSA, and varying conc. of CTP in a total volume of 2.4 ml. The reaction was stopped by adding 3 ml of C:M:HCl (2:1:0.025).


DPM (10^5)
4
3
2
1
0
2
10
20
30
um CTP added

synthesis was increased (Table 14). These results suggest the direct involvement of CTP in TG synthesis. In order to establish the role of CTP in TG synthesis, it was necessary to construct a cell-free system that is deficient in CTP and that fails to generate it (control). To do this, ATP had to be eliminated from the system, and at the same time a way had to be found to allow for optimal incorporation of the marker in TGs. This was done by using ^{14}C-24-CoA (chemically synthesized). By using the acyl-CoA derivative, the requirement for ATP in the cell-free system is minimized.

Cell-free extracts were incubated with ^{14}C-24-CoA, Mg^{++} and other compounds (Table 22). In the control tube (containing ^{14}C-24-CoA and Mg^{++} ions only) incorporation was lower than the tube containing CTP and PA together. The reaction mixture containing CTP showed an increase in incorporation of the label over the control. This may be due to the presence of small amounts of PA which (with CTP) can produce more CDP-DG. When PA alone was included in the reaction mixture, it did not affect synthesis, possibly because of the low levels of CTP. The control incorporated the labeled long-chain fatty acid possibly because of the presence of a residual amount of an intermediate. The intermediate needs to be acylated at the third carbon and can do so in the presence of the long-chain fatty acid--long-chain fatty acids are preferentially added to the third carbon of glycerol. The last column of Table 22 shows the usual system which contains ATP (an indirect source of CTP) and GP (a PA generating system). The counts in this tube were also higher than those found in the control. These results suggest that a compound which

Table 22

Effect of CTP on the Incorporation of Long-Chain Fatty Acid in TGs

Compound	Control (C-24-CoA)* + Mg Ions	Control + 7 um a-GP	Control + 10 um CTP + 5 um PA	Control + 10 um CTP	Control + 5 um PA	Control + ATP + a-GP + Ni Ions
TG	1,168**	1,068	1,993	1,375	1,013	1,624
DG	54	54	84	55	129	64
MG	62	48	237	36	217	52
O	945	1,002	557	686	783	914

* 110 nmoles of ^{14}C-C_{24}-CoA were used

** CPM of the long-chain fatty acid incorporated

is affected by both PA and CTP may be a precursor of TGs. CDP-DG is one such compound. It is synthesized according to this reaction:

PA:CTP acyl transferance

$$PA + CTP \xrightarrow[Mg^{++}]{\text{enzyme}} CDP\text{-}DG + Pi$$

Preliminary attempts to isolate CDP-DG from crude cell-free extracts of M. smegmatis were unsuccessful. Consequently, the cell-free extract was fractioned by centrifugation at 40,000 x g for one hour. The supernatant fraction was discarded, and the pellet fraction was used in the following experiment:

The first tube contained 1.0 ml of the pellet (resuspended in buffer), 5 umoles CTP plus 5 uCi ^{3}H-CTP (Sp. Act. = 500 mCi/mM), 3 umoles PA and 20 umoles $MgCl_2$. The other tube contained the same components as well as all the factors necessary for TG synthesis. After the reaction was allowed to proceed for one hour, the extracted lipids were spotted on Adsorbosil-3 plates and developed in chloroform:methanol:acetic acid: water (50:25:8:3). One cm bands were then scraped off the plate and counted.

This experiment was a duplication of one of Carter's experiments (10) where he isolated radioactivity from bands with an R_f value of 0.60 that co-chromatogramed with authentic CDP-DG (chemically synthesized). He also found that 2.8 nmoles of the added ^{3}H-CTP were incorporated into the lipid. Since authentic CDP-DG was unavailable to us, we had to rely on

the R_f value that Carter reported as our measure.

From the first reaction mixture, radioactivity was isolated from bands with an R_f value of 0.58. Moreover, 2.5 nmoles of the added radioactive CTP were incorporated into the lipid that was isolated from this band. In the second reaction mixture, where optimal conditions for TG synthesis were available, no radioactivity was isolated.

These experiments throw further evidence on the existence and involvement of CDP-DG in lipid synthesis in M. smegmatis. When the conditions were unfavorable for lipogenesis (the first reaction mixture), CDP-DG accumulated and was isolated. However, when conditions for lipid synthesis were optimal (the second reaction mixture), CDP-DG was consumed and was not isolated.

In order to determine the role of CDP-DG in TG synthesis, it was necessary to incubate cell-free extracts with CDP-^{14}C-DG and to trace the labeled DG. Attempts to synthesize CDP-DG chemically were unsuccessful, however. Consequently, this point was not followed any further.

Regulation and Control

The best working cell-free model for TG synthesis was found to require glycerol, GP, FA, ATP, CoA, Mg^{++} and Ni^{++} ions. The requirement for each of the above factors can be justified. Glycerol and FAs are the building blocks of TGs and their importance was discussed earlier. ATP is needed for two processes: the phosphorylation of glycerol and the activation of FAs (to make FA-CoA derivatives) prior to their esterification to glycerol. Magnesium ions are required for the activation of many enzymes such as kinases and CTP:PA transacylase (10, 27), and nickel

ions were found to inhibit the activity of ATPase.

Evidence was presented that TGs are synthesized via the a-GP pathway. If a-GP is an intermediate in the pathway, then adding increasing amounts of this intermediate, in the presence of other factors, should lead to increased synthesis of TGs. This found to be true only for young cells as is shown in Figure 6, but only up to a concentration of 14 umoles. Beyond this concentration, a-GP inhibited TG synthesis. The inhibitory effect of a-GP is more pronounced for all concentrations when cell-free extract from old cells (7 days) were used (Figure 7 and Table 16).

A-GP and CDP-DG are important intermediates in the biosynthesis of lipids in M. smegmatis. A-GP is the first intermediate in the pathway, and without its formation no lipids will be synthesized. CDP-DG, presumably, is the branching point for the synthesis of both neutral and phospholipids. CDP-DG synthesis, is dependent on the concentration of CTP. Consequently, both a-GP and CTP were suspected of being important factors in regulating lipogenesis. To determine the degree of importance of each, lipid synthesis was studied when these two factors were included in the incubation mixture in varying concentrations in relation to each other. As can be seen from Figure 8, CTP exerts a more pronounced effect than a-GP. Without CTP in the incubation mixture, incorporation of the labeled oleic acid in the TGs increased with increasing the concentration of a-GP up to 10.5 umoles, then dropped. On the other hand, including CTP in as a low a concentration as 0.5 umole significantly dropped TG synthesis in all concentrations of a-GP.

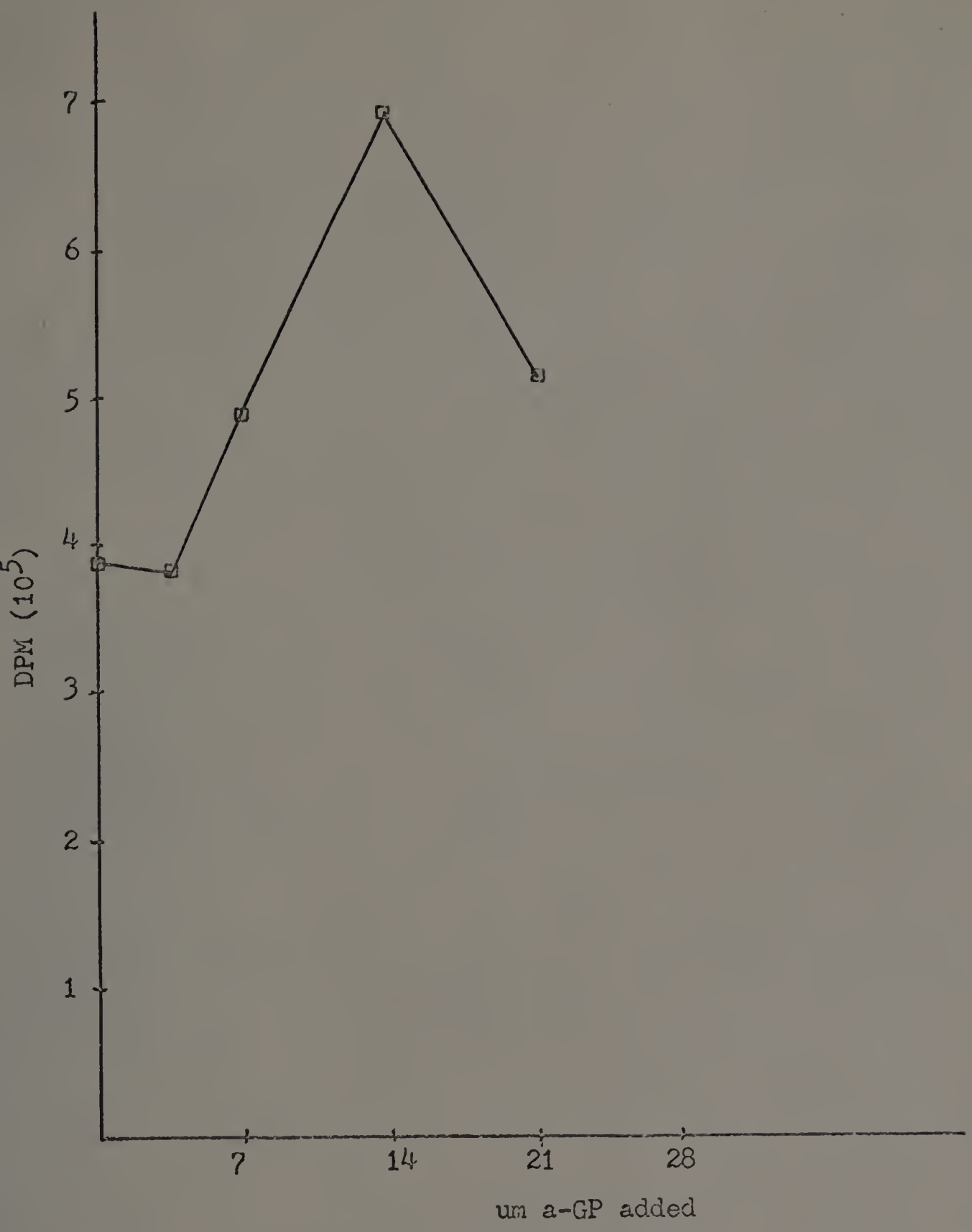


Figure 6

Effect of a-GP Concentration on the Incorporation of ^{14}C-oleic Acid in CFE Prepared From 3-Day Old Cells

Conditions of this experiment were similar to those shown in Figure 5 except that 0.38 uCi oleic acid were used.

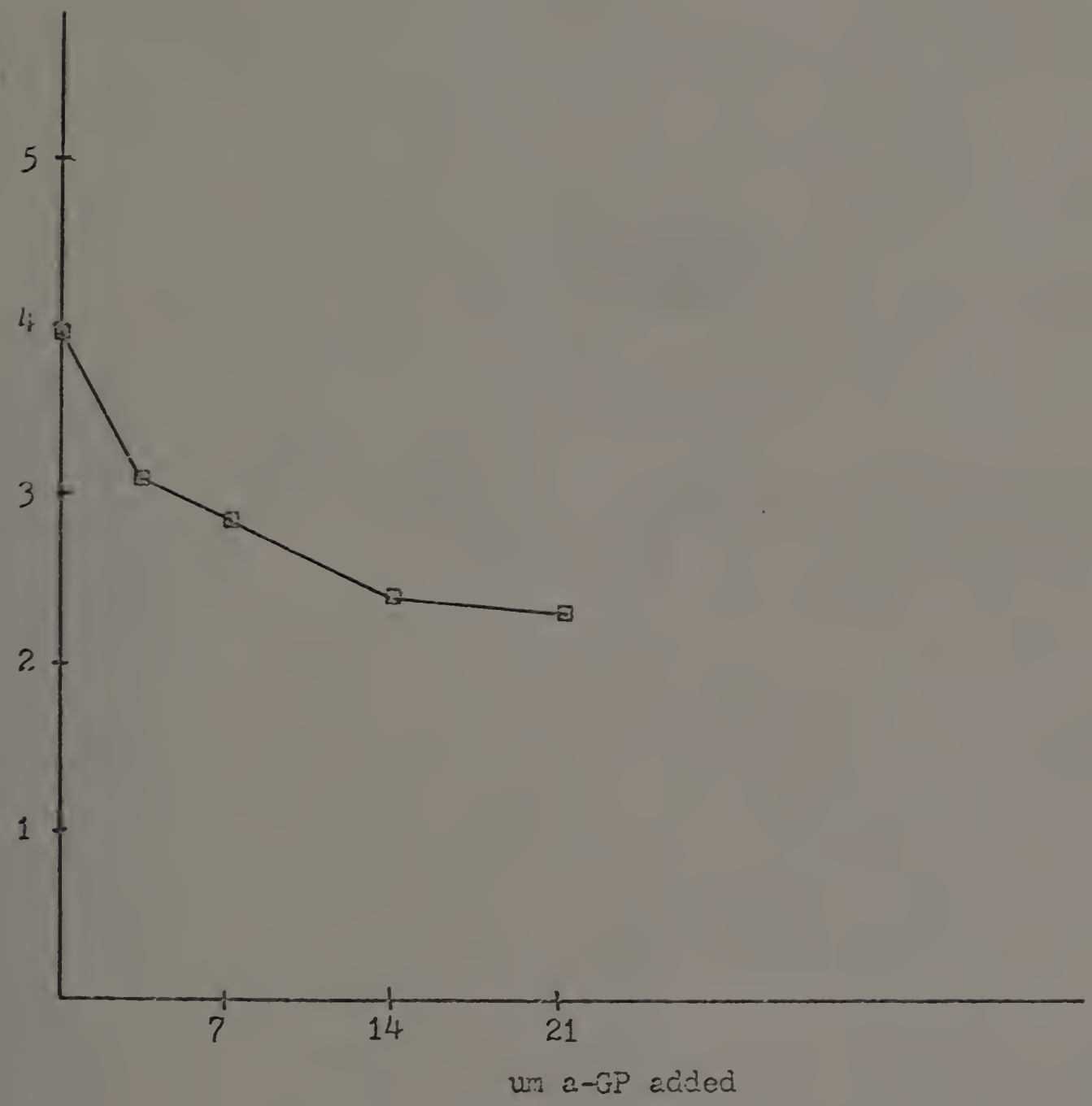


Figure 7

Effect of a-GP Concentration on the Incorporation of ^{14}C-oleic Acid in CFE Prepared From 7-Day Old Cells

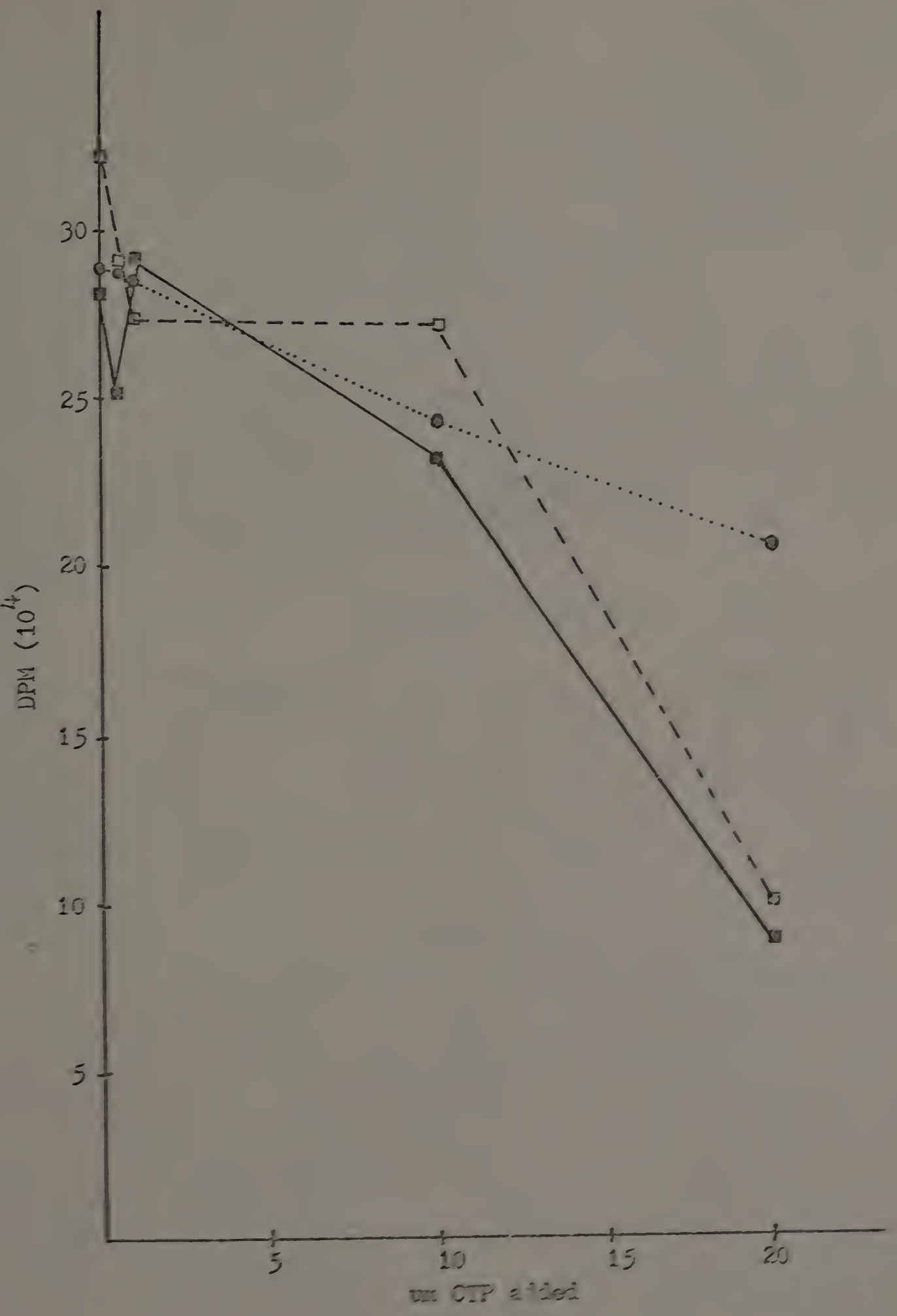


Figure 8

Effect of Varying CTP and a-CF Concentrations on the Incorporation of ^{14}C-citric Acid in CTP; (—●—●—) 0 m a-CF; (-□---□-) 7 m a-CF; (·●···●·) 21 m a-CF. Other conditions are similar to those shown in Figure 5.

Another parameter that was studied in relation to TG synthesis was protein concentration of the cell-free extracts. A concentrated cell-free solution was prepared and diluted with buffer either containint DTT or lacking it. As is seen from Figure 9, the optimal protein concentration for TG synthesis was 12 mgs/ml. Beyond this concentration, no increase in TG synthesis is seen.

Figure 9 shows the incorporation of labeled oleic acid in TGs when the cell-free extract was diluted with buffer containing DTT. At low protein concentrations, incorporation is higher in cell-free extracts containing DTT than in extracts lacking DTT. No significant effect is detected in solutions having a high protein concentration whether DTT is added or not. This can be explained by the fact that at low protein concentrations, there is a lesser amount of enzymes that can be de-activated if oxidized. A reducing agent is needed to protect these enzymes. At higher protein concentrations, the effect of oxidation is not as pronounced because there is an excess of enzymes. Evidently, most of these enzymes were not oxidized and functioned normally.

The results obtained from including DTT in the incubation mixture led to examining the effect of biological reducing agents such as GSH (Figure 10) shows that concentrations of GSH up to 20 umoles had no significant effect on lipid synthesis, but concentrations beyond 20 umoles were extremely inhibitory. At a concentration of 40 umoles there was virtually no incorporation of ^{14}C-oleic acid.

The effect of increasing concentrations of CoA was also examined. Incorporation of ^{14}C-oleic acid was measured at concentrations varying

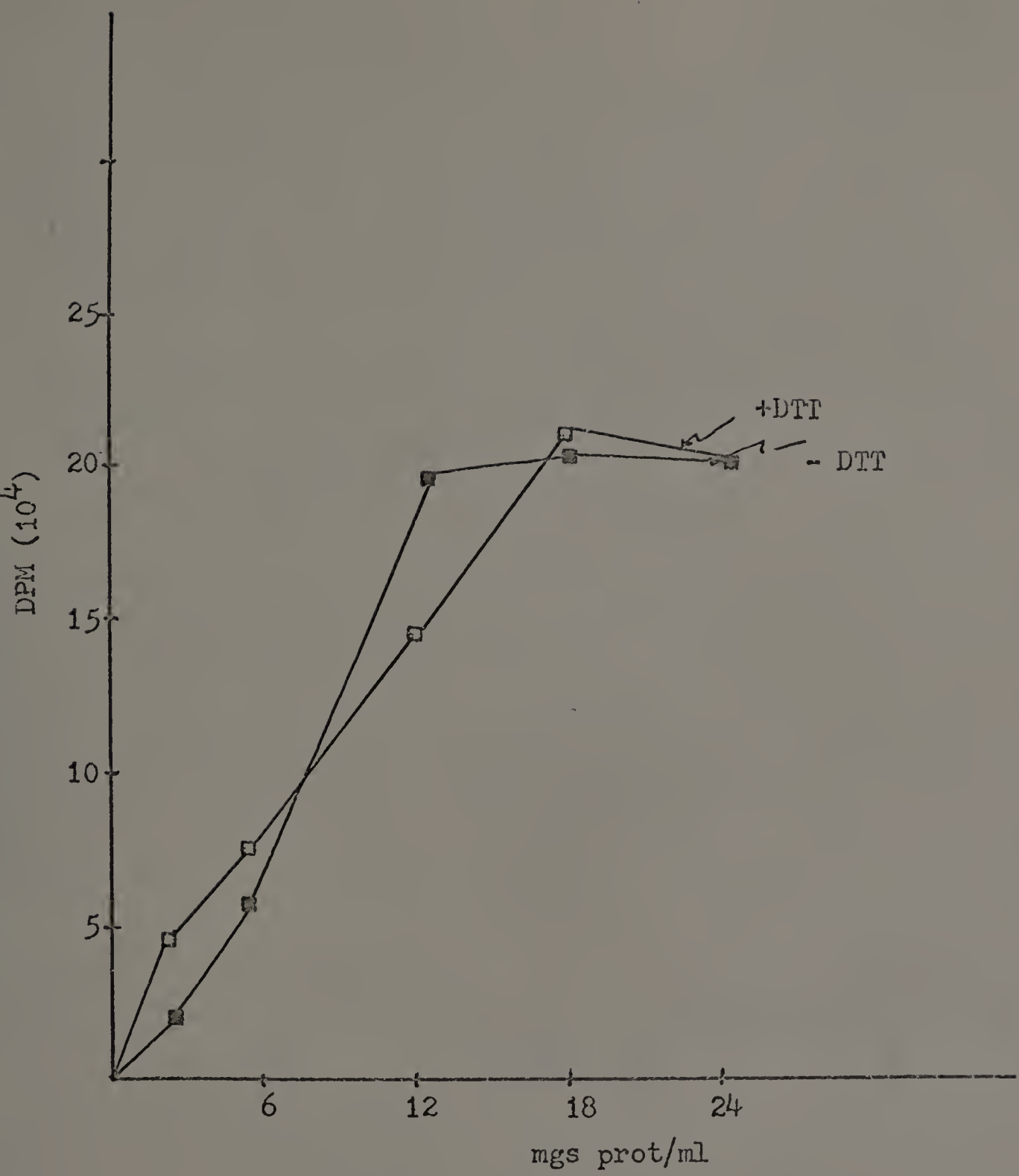


Figure 9

Effect of Protein Concentration on the Incorporation of ^{14}C-oleic Acid in TGs

A concentrated CFE solution was prepared from 3-day old cells and diluted with buffer containing DTT (-□—□-) or with buffer not containing DTT (-▪—▪-). Incubation and other conditions were similar to those shown in Figure 5.

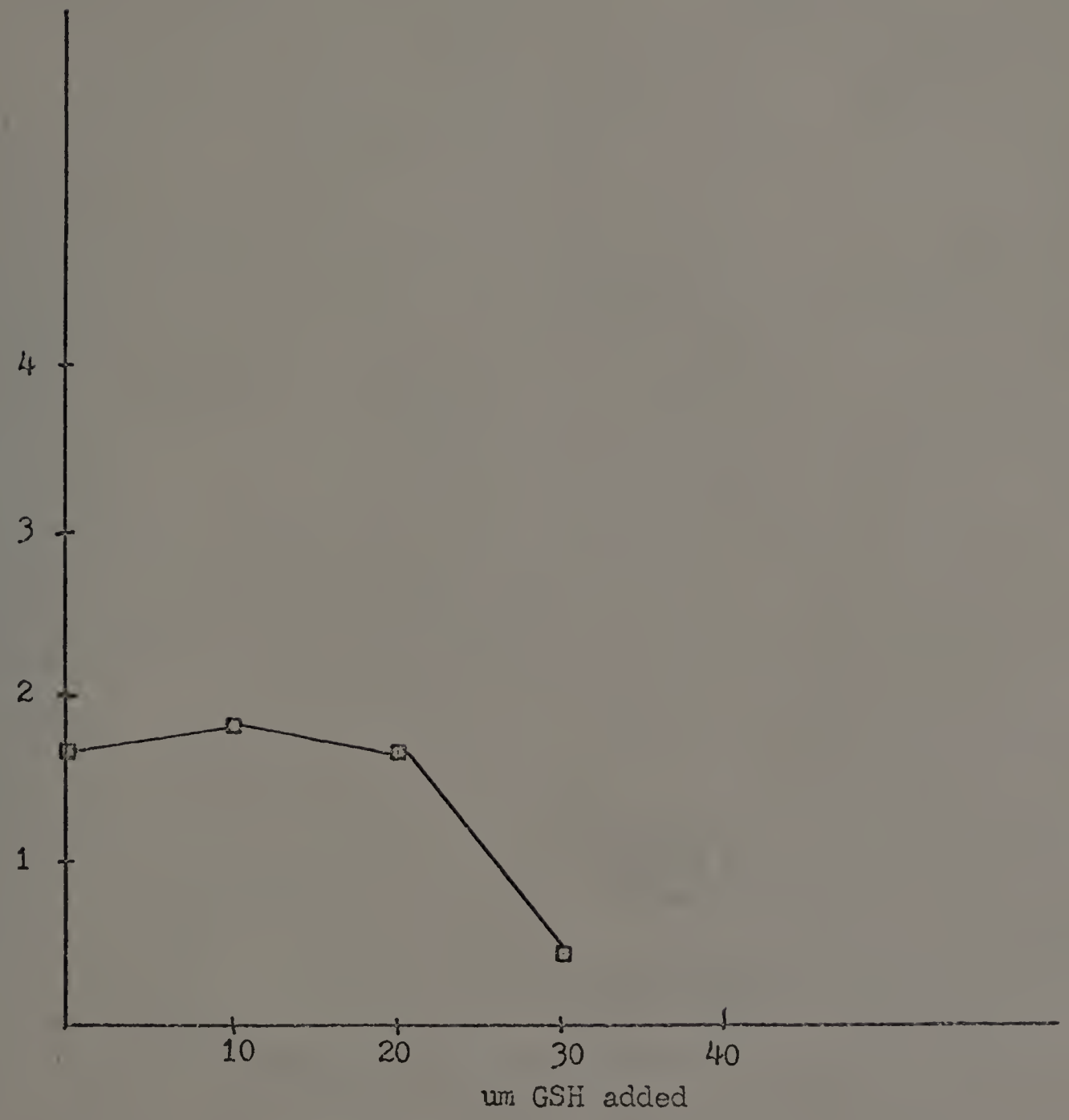


Figure 10

Effect of GSH Concentration on the Incorporation of ^{14}C-oleic Acid

Conditions were similar to those previously described in Figure 5.

from 0 umoles CoA to 0.75 umoles, and the results are shown in Figure 11. The absolute requirement of the system for CoA was previously demonstrated (Table 16). Adding 0.12 umoles to the incubation mixture increased incorporation 5 times. Incorporation of the labeled fatty acid increased in all concentrations of CoA. This experiment describes the importance of CoA in TG synthesis. CoA is necessary, whether the fatty acid is esterified to glycerol as a CoA or ACP derivative.

The effect of varying ATP concentration on TG synthesis is shown in Figure 12. Also shown in the same figure is the effect of including Ni^{++} ions with ATP in the incubation mixtures. Nickel ions complex ATP thus rendering it unavailable to the hydrolytic action of ATPase. Without Ni^{++}, incorporation of ^{14}C-oleic acid into TG was very low because ATPase destroyed the endogenous trace amount of ATP that is present in the cell-free extract. At higher concentrations of ATP, 10-20 umoles, lipogenesis increased whether or not Ni^{++} ions were included in the mixture. Evidently, at such concentrations there is an excess of ATP for TG synthesis. At concentrations above 20 umoles ATP is inhibitory.

Magnesium ions are necessary for activating several enzymes. Figure 13 shows the effect of increasing Mg^{++} ion concentration on TG synthesis. With increasing concentration of Mg^{++}, there is an increase in TG synthesis which levels off at high concentrations. Carter (10) and Cronan (27) have shown that Mg^{++} ions are necessary for the action of CTP: PA transacylase, the enzyme involved in the synthesis of CDP-DG. Because many enzymes involved in TG synthesis require divalent cations

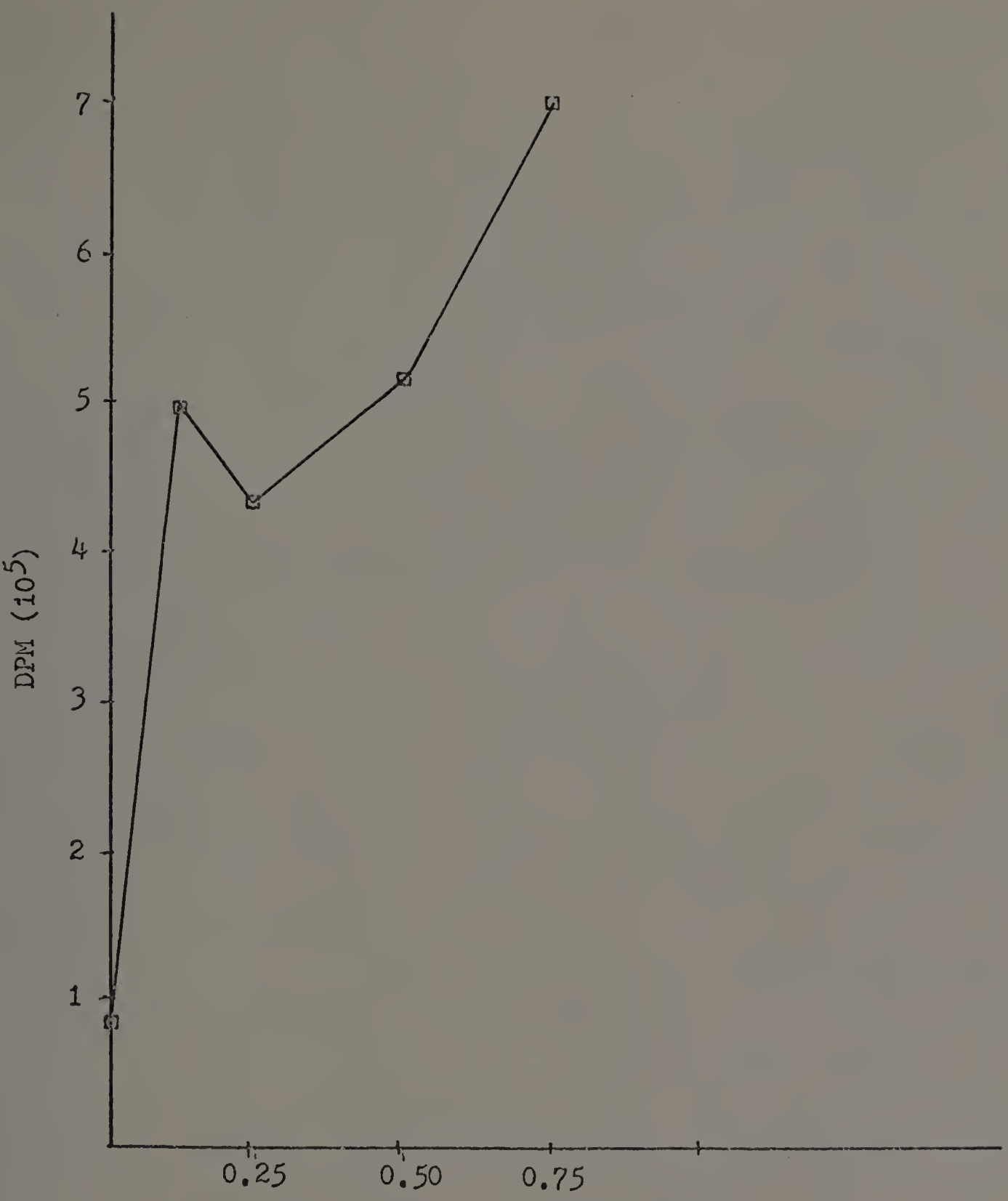


Figure 11

Effect of CoA Concentration on the Incorporation of ^{14}C-oleic Acid in TGs

Conditions were similar to those shown in Figure 5 except that 0.38 uCi oleic acid were used.

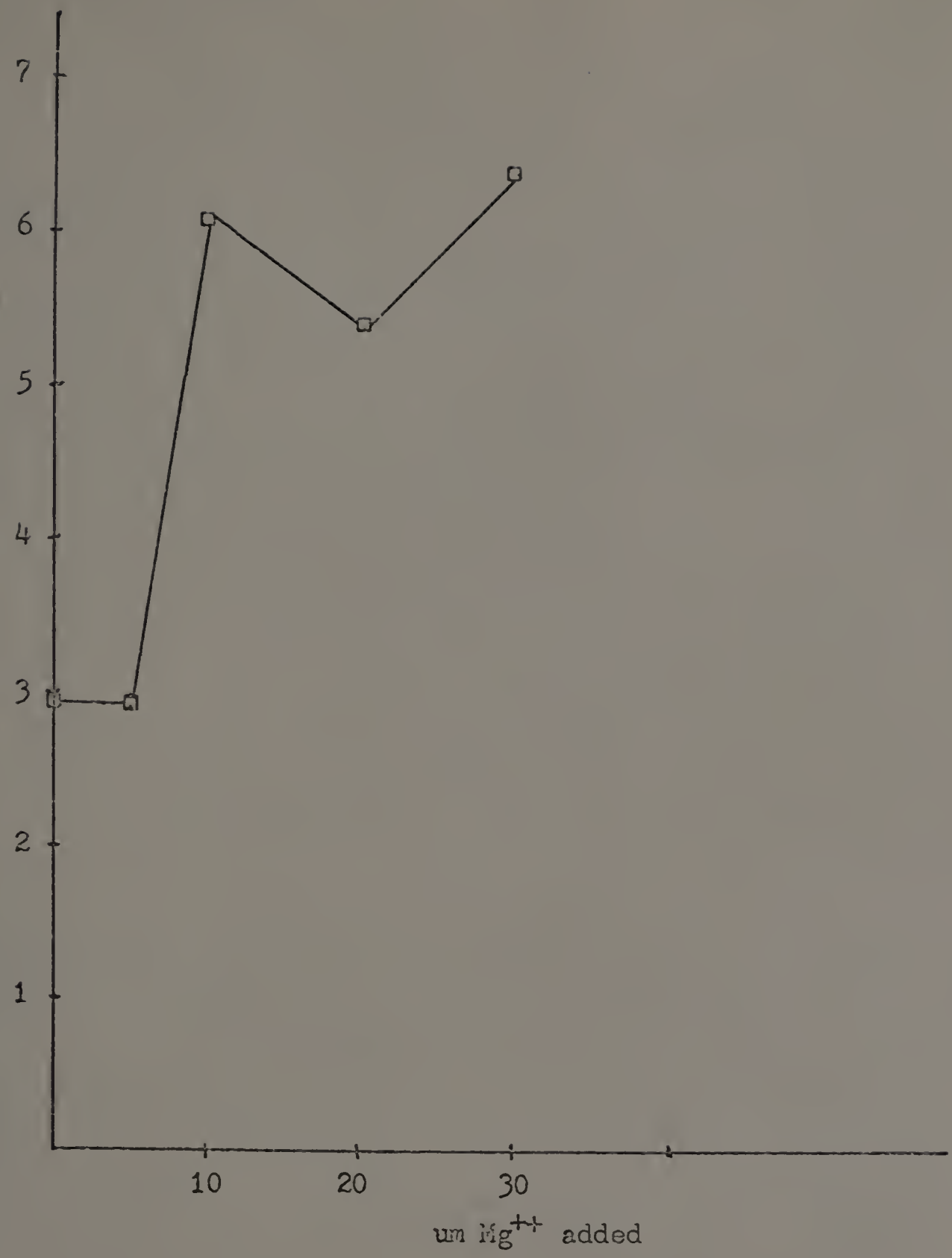


Figure 13

Effect of Magnesium Ion Concentration on the Incorporation of ^{14}C-oleic Acid in TGs

Conditions are similar to those shown in Figure 11.

like Mg^{++} and Mn^{++}, the effect that Mg^{++} exerts is expected.

The effect of incubation time on TG synthesis was also examined. As is shown in Figure 14, there is a linear increase of TG synthesis with increasing time. This can only mean that there is continuous synthesis of TGs. It cannot be addition of the labeled FA on the third carbon of glycerol. If the latter was the case then there would be a sharp rise in TG synthesis in a short time followed by a drop. With continuous de novo synthesis there is continuous synthesis of TGs and hence the linear relationship with time.

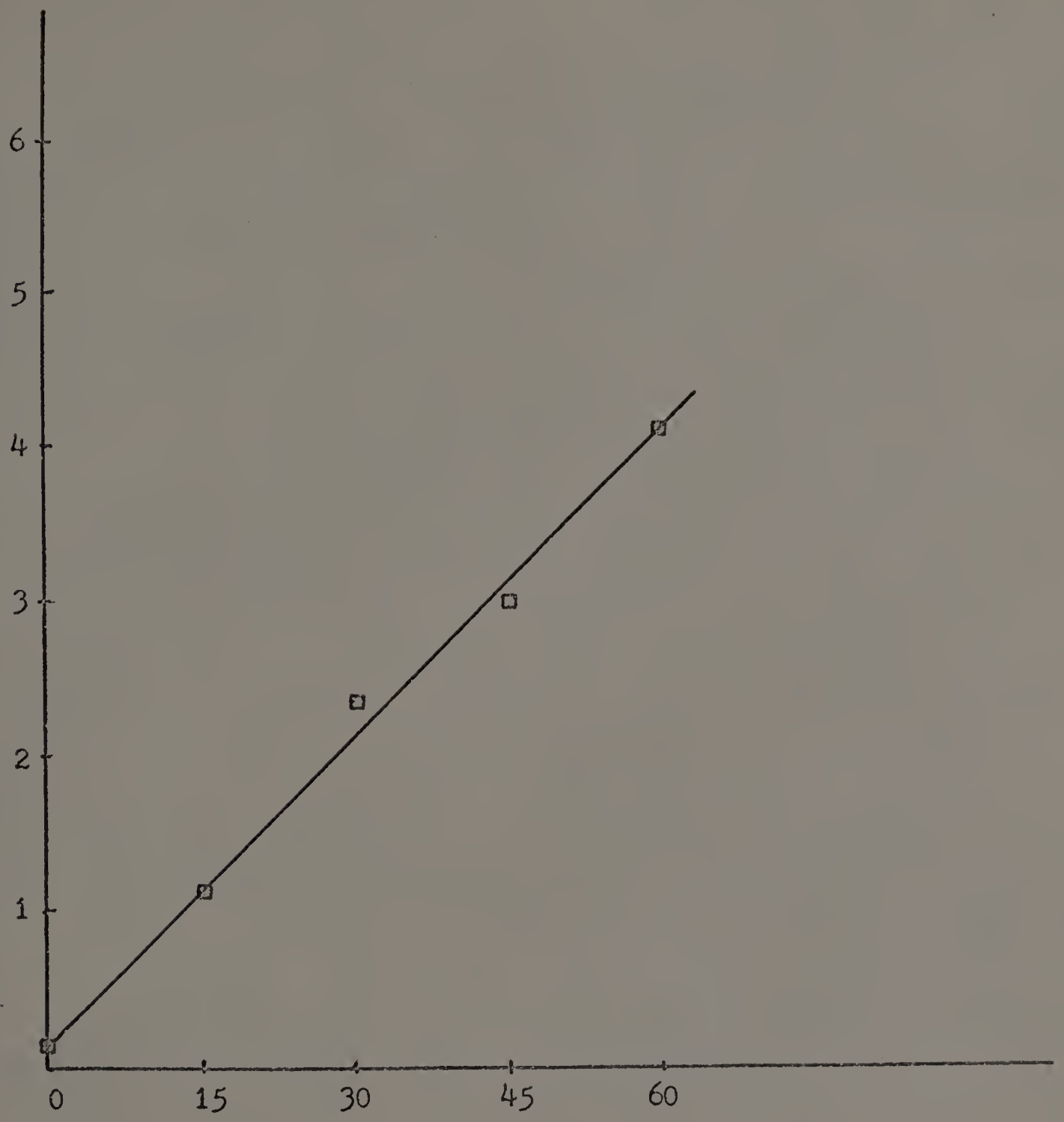


Figure 14

Effect of Incubation Time on the Incorporation of ^{14}C-oleic Acid in TGs

DISCUSSION

Mycobacterium smegmatis is unique among bacteria in its ability to synthesize relatively high amounts of lipids (approximately 20% of the total dry weight), half of which are TGs. Whether or not the organism exhibits the same pattern of synthesis in its natural habitat is not known. But since these compounds constitute such a high percentage of the weight of the organism under the culturing conditions employed, they may be of some importance to the cell. This study was undertaken in an attempt to verify the structure, function, biosynthesis and regulation and control of TGs in this organism.

From the studies of the structure of TGs [which appeared as part of a publication by Walker et. al. (69)], it was found that these compounds exhibit a defined, non-random pattern in the nature and distribution of fatty acids on the glycerol molecule. Furthermore, the structural analysis of the phospholipids from M. smegmatis shows that the FAs esterified to the phospholipids are closely related to the FAs of the TGs. In both compounds, the a-carbon is mainly esterified with $C_{18:1}$ (in TGs) or a derivative of it ($C_{19\ Branched}$ in phospholipids) and the B-carbon is esterified with $C_{16:0}$ or a derivative of it ($C_{17\ Branched}$). Branching of fatty acids was reported to occur while the FA is attached to a phospholipid molecule. Because of such similarity in structure, both the TGs and phospholipid may be derived from a common intermediate. This implies that synthesis of phospholipids and TGs proceeds via the same pathway.

Apparently, Youman's medium, in which M. smegmatis thrives best, plays a major role in determining the final concentration of TGs in the cell. Youman's medium has a high carbon content: glycerol, asparagine and citrate. All of these compounds are easily metabolizable serving as sources of energy and carbon. Evidently, there is some selectivity involved in TG synthesis. Asparagine and citrate, after entering the cell, can enter the tricarboxylic acid cycle with little expenditure of energy, and can be metabolized to the acetyl-CoA level. At this level, the two-carbon units can be used to synthesize new fatty acids, the nature of which will be determined by the need of the cell. Glycerol is structurally fit for the synthesis of both neutral and phospholipids. Thus it can serve as a backbone molecule for the addition of the FAs synthesized from the other carbon sources. This might explain the little variation in the amount of TGs synthesized when the cells were grown in different glycerol concentrations. The amount of glycerol that was utilized per gram of cells, and the TG content of the cells were constant regardless of the glycerol concentration in the culture medium. If glycerol was used as a source of energy, then its utilization should be proportional to its concentration in the medium. This was not the case. Only the amount needed to build complex molecules (TGs and phospholipids) was consumed.

Evidence for the validity of the above hypothesis was obtained from experiments with cell-free extracts (Tables 18 and 20). When the cell-free extract was incubated with ^{3}H-glycerol alone, there was little incorporation of the labeled glycerol in the TGs. Incubating cell-free

extracts with ^{14}C-FAs showed high incorporation of the isotope in the TGs. This high incorporation could be achieved because of the presence of excessive amounts of glycerol in the cell-free extract since the cells were grown in Norman's medium. When the cell-free extract was incubated with both ^{3}H-glycerol and ^{14}C-oleic acid, however, incorporation of both isotopes was high. These experiments show that glycerol can be incorporated into TGs only if there is an ample supply of fatty acids. In other words, the fatty acids are the limiting factor of TG synthesis in cell-free extracts.

Triglycerides are inert compounds that do not have a significant role either structurally or functionally. From the studies of the structural analysis of these compounds, it was found that long-chain fatty acids ($C_{>16}$), which are esterified to the third carbon, constitute 10% of the total fatty acids esterified to that carbon (69). Presumably these long-chain fatty acids are stored on the TGs to be donated to cyclic acids (2), which are constituents of cell walls. Furthermore, evidence of the role of TGs as storage products was obtained from culturing experiments. When the cells were grown in media containing increasing concentrations of nutrients, the TG content increased proportionately. The relationship between TG content and abundance of nutrients was found to be true in experiments with cell-free extracts as well. Incubating cell-free extracts in excessive amounts of glycerol and increasing concentrations of fatty acids increased TG synthesis proportionately. These experiments suggest that TGs are storage products analogous to poly-β-hydroxybutyrate and glycogen in bacteria.

If TGs are truly storage products, then they should be consumed if the cells were starved. This was found to be true. When cells, having TGs labeled with ^{14}C-oleic acid, were incubated in a carbon-free medium for different time intervals, the amount of radioactivity in the TGs dropped but simultaneously increased in the phospholipids up to a certain point then levelled off (Figure 2). In the carbon-free medium, the fatty acids may have been hydrolyzed from the TGs (decrease in TGs) and added intact to the more important structural and functional phospholipids. There is also the possibility that after the fatty acids were hydrolyzed from the TGs, they were degraded via B-oxidation to yield energy and 2-carbon units. These carbon units were then incorporated into the phospholipids. The levelling of the curve may be due to exhaustion of the carbon and energy sources which resulted in a halt of phospholipid synthesis.

These experiments seem to prove that TGs are storage products synthesized when there is an excess of nutrients and consumed when the need arises.

Biosynthesis of Trigylcerides

Two pathways have been shown to be responsible for TG synthesis in higher forms: the MG pathway and the a-glycerophosphate pathway. In the MG pathway, the glycerol molecule is esterified with three acyl-CoA derivatives in a stepwise fashion to produce a MG, a DG and finally a TG molecule. Prior to their esterification to glycerol, fatty acids have to be activated by forming the acyl-CoA derivative, a step that requires the expenditure of energy. Once the monoglyceride molecule is

synthesized, however, it is incorporated intact into the TGs. One way of checking whether the MG pathway operates in a certain system is to supply that system with a monoglyceride labeled with two isotopes, where the ratio of counts of the isotopes is known, then recovering the same ratio in the DGs and TGs (28). Alternatively, verification of the existence of the MG pathway could be achieved by examining the effect of increasing concentrations of MG on TG synthesis. If the pathway operates then increasing the concentration of MGs should result in an increase of TG content.

The two approaches were followed in order to see if the MG pathway operates or significantly contributes to TG synthesis in M. smegmatis. When cell-free extracts were incubated with doubly labeled MG, the ratio of counts of $^{3}H/^{14}C$ in the TGs was higher than the ratio of counts in the added MG (Tables 3 and 4). Even the unreacted MGs isolated after the reaction was stopped had a higher ratio of counts than the original MG. This implies that counts contributed by the ^{14}C-fatty acid are decreased probably because the fatty acids are hydrolyzed from the MG. MG lipase was found to be an active enzyme in other organisms and was shown to have a higher catalytic activity on the MGs than on the DGs or TGs. Furthermore, evidence of the activity of the MG lipase is shown in the high counts recovered in the FA fraction.

In order to minimize this hydrolytic activity, the crude cell-free extract was fractionated into pellet and supernatant fractions. When the doubly labeled MG was incubated with either the pellet or the supernatant fraction, the ratio of counts in the TG fraction was also altered (Table 5).

Evidence of the non-involvement of MGs in TG synthesis was also obtained when the cell-free extract was incubated with MG and other factors and no increase in incorporation of labelled fatty acids was detected (Table 5). From these results, it is evident that the MG pathway does not contribute to TG synthesis in M. smegmatis. Furthermore, monoglycerides are not intermediates in the pathway that leads to TGs; otherwise, addition of MGs should increase TG synthesis. The fact that MGs were isolated from whole cells implies that they arise probably as a result of dephosphorylation of lysophosphatidic acid. As to why they are synthesized is still to be explored.

The structural similarity of the phospholipids and TGs indicates that these compounds may be derived from the same intermediate. This implies that one pathway is involved in their synthesis. The only pathway from which both phospholipids and TGs could be derived is the a-glycerophosphate pathway. Preliminary evidence of the existence of this pathway in M. smegmatis was obtained when whole cells were pulse-labelled with ^{14}C-glycerol and counts were recovered in a-GP, PA and TGs (Table 6). The recovery of counts in these fractions showed that a-GP and PA are intermediates in the synthesis of TGs. Further evidence of the direct involvement of a-GP was obtained when there was an increase in TG synthesis upon adding a-GP to a cell-free mixture containing the factors necessary for TG synthesis (Table 10). Increasing the concentration of a-GP in a fortified cell-free system from young cells (i.e., a cell-free system supplied with all the factors necessary for optimal TG synthesis) produced a proportional increase in TG synthesis (Figure 6).

A reversal effect was obtained when cell-free extracts from old cells were incubated with increasing amounts of a-GP; i.e., with increasing concentrations of a-GP there was a proportional decrease in TG synthesis.

The fact that a-GP stimulated TG synthesis in CFE from young cells but depressed it in cell-free extracts from old cells could be related to the difference in the rate of metabolism between the young and old cells. When the cells are young, they actively metabolize the nutrients that are supplied to them in order to make new compounds. Addition of intermediates that are precursors to these compounds should stimulate their synthesis. With age, however, there is a drop in the metabolic activity of cells and a concurrent accumulation of intermediates that may lead to repression of the enzymes involved in the metabolism of these intermediates. A-glycerophosphate might have caused such a repression when it was incubated with cell-free extracts from old cells. It is also possible that a-GP was not utilized efficiently in these cell-free preparations because the enzymes involved in its metabolism were inhibited by the end-products (Phospholipids and TGs).

Lysophosphatidic acid is presumably the second intermediate in the a-glycerophosphate pathway. Lands (personal communication) showed that the rate of acylation of lyso-PA is ten times faster than that of a-GP. This high rate of turnover coupled with the fact that no system has been found to isolate lyso-PA, made isolating it very difficult. Indirect evidence, however, for its synthesis and involvement in the a-glycerophosphate pathway in M. smegmatis was obtained from several experiments. In the experiments performed to verify the involvement of MG pathway

and the effect of MGs on triglyceride synthesis, the MGs are found to be ineffective in stimulating such synthesis. However, MGs were isolated, though in small quantities, from whole cells and all the experiments with the cell-free extracts. Since MGs are not involved in TG synthesis, they can only arise as by-products of certain reactions like lipase hydrolysis of DGs and TGs and the dephosophorylation of lysophosphatidic acid. Evidence for the occurence of the last reaction (i.e., the dephosphorylation of lyso-PA) was obtained when a parallel experiment using ^{14}C-palmitic acid and ^{14}C-oleic acid as markers, was performed. The MGs isolated from the two reaction mixtures showed differences in their labeling. Only the MGs isolated from the reaction mixture containing labeled palmitic acid were labeled, whereas those isolated from the reaction mixture containing ^{14}C-oleic acid were not (Table 11). If these MGs were derived from the action of lipases on DGs and TGs, they should have been labeled with ^{14}C-oleic acid as well, since oleic acid was shown to be acylated to all three carbons in the cell-free extracts (Table 21). If the MGs were produced from the action of lyso-PA phosphatase on lyso-PA, however, only the lyso-PA with the proper configuration that fits as a substrate for the action of the enzyme will be acted on. Two-monopalmitin glycerophosphate is such a substrate and hence was dephosphorylated to produce monopalmitin. Palmitic acid was proved to be preferentially acylated to the B-carbon (69). These experiments demonstrate that a-GP is acylated with palmitic acid to yield 2-monopalmitin from which MGs arise as by-products. One-monooleyl glycerophosphate is not a substrate for the catalytic activity of lyso-PA

phosphatase and hence cannot be dephosphorylated to form monoolein. This explains the absence of counts from the reaction mixture containing oleic acid as a marker.

Evidence of PA being the third intermediate in the pathway was first obtained when whole cells were pulse-labelled with ^{14}C-glycerol and high counts were recovered from the PA fraction (Table 6). Other experiments (Tables 7 and 8) show that PA has a high rate of turnover suggesting that it plays a major role in the synthesis of lipids. Indirect evidence of the involvement of PA in TG synthesis was obtained when detergents--whether anionic, cationic or netural--were included in the reaction mixtures and there was a drop in TG synthesis (Table 11). Detergents are assumed to inhibit the enzyme PA phosphohydrolase (59) which hydrolyzes the phosphate from PA to yield 1,2-DG. These experiments suggest that PA is an intermediate in the pathway and is a precursor to 1,2-DGs from which TGs are derived. However, when PA was added to the incubation mixture with all the factors necessary for optimal TG synthesis, TG synthesis decreased. Addition of PA and CTP to the same mixtures stimulated TG synthesis (Table 14). It seems that PA exerts a repressive activity on the enzymes that are involved in its metabolism when it is found in excessive amounts. This repression could be relieved if the excess PA could be channeled through other reactions. One reaction where PA could be consumed is the synthesis of CDP-DG which requires CTP. Apparently, CTP is found in limiting concentrations in the cell-free extracts, or an active CTPase may destroy the excess CTP when a cell-free extract is prepared. Consequently, when CTP was

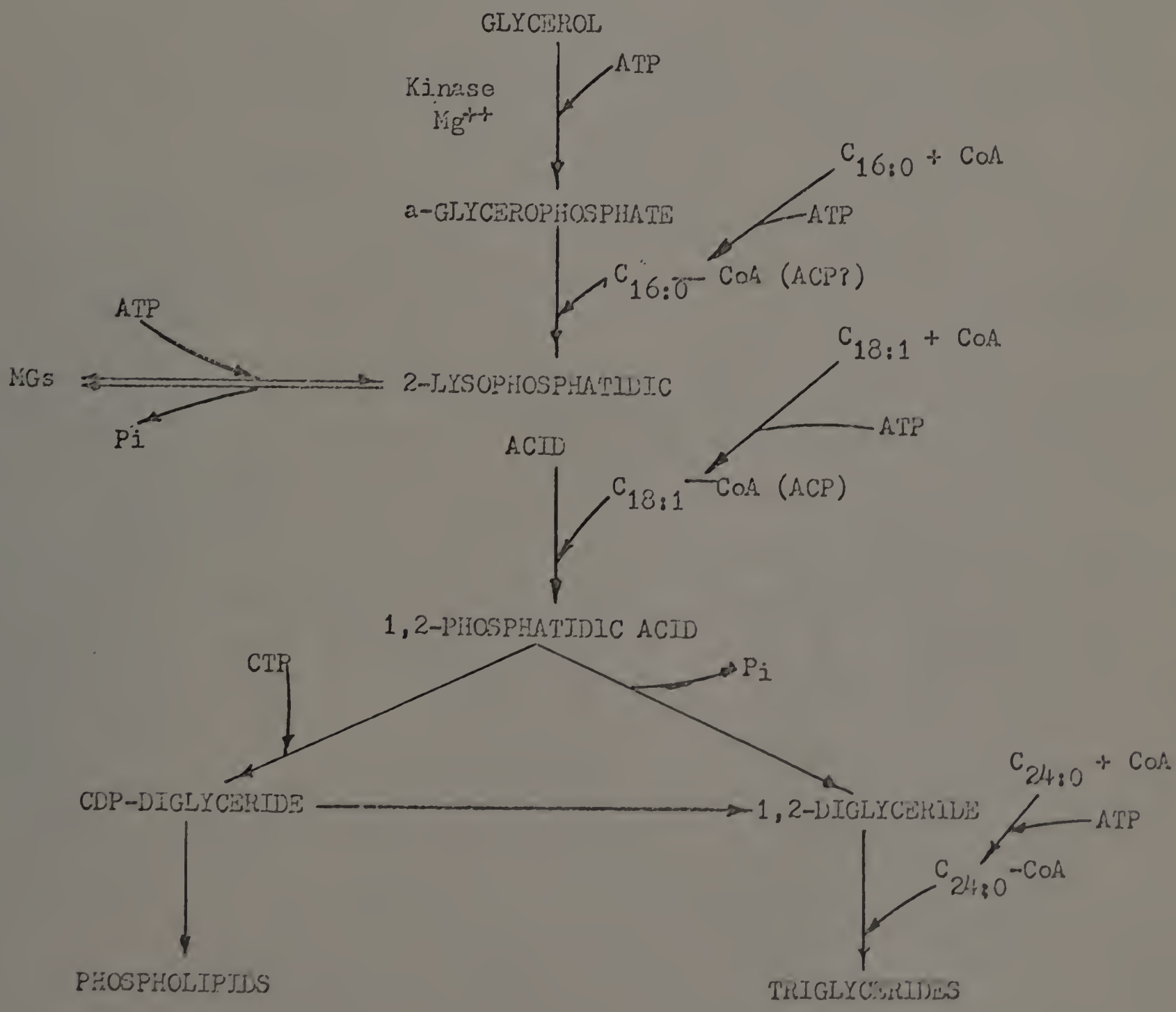
supplied to the incubation mixture, the excess PA was consumed and TG synthesis was increased. The increase in TG synthesis is probably due to the formation of CDP-DG which can act as another source of 1,2-DGs. This suggests that CDP-DG may be another intermediate in the synthesis of TGs. Hydrolysis of CDP-DG yields CDP and 1,2-DG, which can be acylated at the third carbon to yield TGs. Existence of CDP-DG was confirmed when it was isolated in concentrations equivalent to those obtained by Carter (10) when his experiment was duplicated. The effect of adding CDP-DG on TG synthesis was not examined because attempts to synthesize it chemically were unsuccessful.

In certain experiments (Tables 14 and 15) inclusion of PE isolated (by TLC) from M. smegmatis in the reaction mixture enhanced TG synthesis. However, PE from other sources and other preparations (purified on DEAE column) was found to be ineffective. The stimulatory effect that was obtained when the first batch of PE was used could be due to the contamination of the compound with some factors that enhance TG synthesis. More purified samples did not show the same effect.

The last intermediate in the pathway is 1,2-DG. This compound could be derived from PA (by the action of PA phosphohydrolase) or from the hydrolysis of CDP-DG. In order that DGs go to TGs, they have to be in the proper configuration. Only 1,2-DGs were effective in stimulating TG synthesis when they were included in the reaction mixture (Table 14). DGs with 1,3- configuration did not significantly affect synthesis. The little stimulatory effect that was observed when 1,3-DGs were added could be due to their contamination with 1,2-DGs (the DG preparation was

approximately 85% 1,3-DGs and 15% 1,2-DGs). This could be due to the specificity of the enzymes involved in acylating the DGs to produce TGs. Unless these DGs are of the 1,2- configuration, they will not be used as a substrate for the acylation reaction.

From these data the following pathway leading to TG synthesis is proposed:



From the lipase hydrolysis experiments and from previous analyses, it is believed that there is de novo synthesis in the cell-free extracts that proceeds via the mentioned pathway. Palmitic acid is first esterified to the second carbon of a-GP, then oleic acid is added at the first carbon. The details of esterification of these two fatty acids are beyond the scope of this work, but preliminary evidence suggests that the ACP derivatives of these fatty acids might be the form in which these compounds exist before they are esterified. Inclusion of crude ACP prepared from M. smegmatis in the reactin mixture increased TG synthesis (Tables 14 and 15). More conclusive evidence could be obtained if more purified ACP could be isolated and attached to ^{14}C-palmitic and ^{14}C-oleic. The effect of these compounds on TG synthesis then can be examined.

Studies on the regulation and control of TG synthesis were attempted, but further work is needed to elucidate the intricate mechanisms that are involved in such a synthesis. As was presented, both a-GP and CTP are major contributors to the control of TG synthesis. Other factors such as ATP, CoA, Mg^{++} ions and Ni^{++} ions play a significant role in the process. The need of the system for these factors is illustrated in the pathway, and was previously covered in Results.

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