712370

946
(R)

Oak Ridge
Associated
Universities

February 26, 1980

Mr. Kenneth M. Haythorn, Director
Energy Programs and Support Division
Department of Energy
Oak Ridge, Tennessee 37830

Subject: GRANT APPLICATION ENTITLED *LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA*

Dear Mr. Haythorn:

Enclosed are three copies of a grant application to NIH for a new project entitled *Lipid Metabolism in TPA-Differentiating Leukemia*. Draft copies of this grant, under the title *Ether Lipid Metabolism in Cell Transformation*, were forwarded to your office for review on January 14, 1980, and approval for transmittal to NIH was given by Dr. Benson on February 21.

The proposed research will be carried out under policies and procedures previously established between ORAU and DOE and will be supervised by Dr. Myles Cabot.

We will keep you advised concerning the status of the grant.

Sincerely,

William E. Fitling

FOR Philip L. Johnson
Executive Director

RYAN:br

Enclosures

REPOSITORY Oak Ridge Operations Records Holding Area
COLLECTION Documents 1944-94
BOX No. B-87-9 Bldg. 2714-H
FOLDER 20-78-80 NIH

1079484

2. 1/12

Oak Ridge
Associated
Universities

Post Office Box 117
Oak Ridge, Tennessee 37830
Telephone 615 576-3300

Executive
Office

February 26, 1980

Division of Research Grants
National Institutes of Health
U. S. Public Health Service
Bethesda, Maryland 20205

Gentlemen:

We are submitting for your consideration seven copies of a grant application entitled *Lipid Metabolism in TPA-Differentiating Leukemia*. This project will be supervised by Dr. Myles Cabot.

Oak Ridge Associated Universities is a nonprofit corporation sponsored by 50 Southern colleges and universities. The major portion of its activities are carried out under a long-term operating contract with the U. S. Department of Energy. Certain conditions arising from this relationship between ORAU and the DOE are set forth under Section E of the application.

If questions should arise during the review of this proposal, please do not hesitate to call Dr. Cabot at area code 615, 576-3122.

Sincerely,

Original Signed By
PHILIP L. JOHNSON

Philip L. Johnson
Executive Director

br

Enclosures

bcc: Mr. Kenneth M. Haythorn DOE ORO (3)
Executive Office (2)
W. F. Countiss
John T. Crockett
Myles Cabot

1079485

SECTION I

Form Approved
O.M.B. 68-R0249

DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

LEAVE BLANK		
TYPE	PROGRAM	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL (Month, Year)		DATE RECEIVED

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 15A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)
LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)
CABOT, Myles C.

2B. TITLE OF POSITION
Biochemist

2C. MAILING ADDRESS (Street, City, State, Zip Code)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

2D. DEGREE
Ph. D.

2E. SOCIAL SECURITY NO.
[redacted]

2F. TELEPHONE DATA — Area Code: 615 — TELEPHONE NUMBER AND EXTENSION: 576-3122

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)
Biological Chemistry

2H. MAJOR SUBDIVISION (See Instructions)
Medical and Health Sciences Division

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application
FROM: December 1, 1980
THROUGH: November 30, 1983

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3: $216,650

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD: $67,750

6. PERFORMANCE SITE(S) (See Instructions)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

Third Congressional District

7. Research Involving Human Subjects (See Instructions)
A. [X] NO B. [ ] YES Approved: _____ Date
C. [ ] YES – Pending Review

8. Inventions (Renewal Applicants Only - See Instructions)
A. [X] NO B. [ ] YES – Not previously reported
C. [ ] YES – Previously reported

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 15B)

9. APPLICANT ORGANIZATION(S) (See Instructions)
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

(IRS No. 62-0476816)

Third Congressional District

10. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)
Philip L. Johnson
Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830
Telephone Number (s) 615 576-3300

11. TYPE OF ORGANIZATION (Check applicable item)
[ ] FEDERAL [ ] STATE [ ] LOCAL [X] OTHER (Specify)
Not for profit, private institution

12. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830
Telephone Number 615 576-3056

13. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)
20 Other (Medical and Health Sciences Division)

14. ENTITY NUMBER (Formerly PHS Account Number)
1620476816A1

15. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Per" signatures not acceptable)	A. SIGNATURE OF PERSON NAMED IN ITEM 2A	DATE
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 10 — Philip L. Johnson	DATE 2-26-80

PHS-398 (Formerly NIH-398)
Rev. 1-73

1079486

SECTION 1

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

RESEARCH OBJECTIVES

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION

Oak Ridge Associated Universities, P. O. Box 117,
Oak Ridge, Tennessee 37830

NAME, SOCIAL SECURITY NUMBER, OFFICIAL TITLE, AND DEPARTMENT OF ALL PROFESSIONAL PERSONNEL ENGAGED ON PROJECT, BEGINNING WITH PRINCIPAL INVESTIGATOR

Myles C. Cabot [redacted] Principal Investigator

TITLE OF PROJECT

LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

USE THIS SPACE TO ABSTRACT YOUR PROPOSED RESEARCH. OUTLINE OBJECTIVES AND METHODS. UNDERSCORE THE KEY WORDS (NOT TO EXCEED 10) IN YOUR ABSTRACT.

The potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to stimulate phospholipid metabolism in mouse epidermis and in a variety of cells maintained in culture. When used at very low concentrations, TPA also induces differentiation in human leukemic cells (HL-60). We have shown that TPA elicits stimulation of phosphatidylcholine metabolism in leukemia cells and that this stimulation preceeds the expression of the various differentiation markers. However, the mechanism by which TPA alters phospholipid metabolism and the role of phosphatidylcholine in the cell differentiation process remains obscure. Essentially no information is available at the molecular level regarding the effect of TPA on the enzymes and cofactors responsible for phosphatidylcholine synthesis. By utilizing tumor promoters, such as TPA, we will be able to correlate the dynamics of phospholipid metabolism with cellular differentiation. Total lipids isolated from control and TPA-exposed cells will be rigorously analyzed, and the activities of the key enzymes of phosphatidylcholine synthesis (choline kinase, CTP-phosphocholine cytidylyltransferase, CDP-choline:1,2-diacylglycerol phosphocholinetransferase) will be characterized in vitro. Additionally, TPA may modify phosphatidylcholine metabolism by altering the levels of intracellular cofactors and precursors. The levels of CTP, phosphocholine, choline, CDP-choline, and diacylglycerol in control and treated cells will be determined. The assimilation of radiolabeled fatty acids into phosphatidylcholine and de novo synthesis from glucose and glycerol will in turn be investigated. The ultimate objective is to understand what factors are responsible for the TPA-induced stimulation of phosphatidylcholine turnover in differentiating cells.

LEAVE BLANK

1079487

SECTION II – PRIVILEGED COMM CATION

DETAILED BUDGET FOR FIRST 12-MONTH PERIOD

FROM 12/01/80 THROUGH 11/30/81

DESCRIPTION (Itemize)		TIME OR EFFORT %/HRS	AMOUNT REQUESTED (Omit cents)		
PERSONNEL					
NAME	TITLE OF POSITION		SALARY	FRINGE BENEFITS	TOTAL
Cabot, Myles C., Ph.D.	PRINCIPAL INVESTIGATOR	50	12,120	2,365	14,485
Borman, Linda, Ph.D.	Cell Biologist	15	3,580	715	4,295
Welsh, Clement J.	Biochemist	70	10,730	2,415	13,145
TO BE NAMED	Postdoctoral	100	13,000	1,300	14,300
TO BE NAMED	Research Technician	50	7,000	1,575	8,575
			46,430	8,370	54,800

CONSULTANT COSTS

EQUIPMENT Waterbath Shaker $950 — 950

SUPPLIES
- Tissue Culture Ware 2,000
- Cell Culture Media & Serum 3,500
- Chemicals 1,500
- Radiolabeled Compounds 4,000

11,000

TRAVEL
- DOMESTIC 1,000
- FOREIGN

PATIENT COSTS (See instructions)

ALTERATIONS AND RENOVATIONS

OTHER EXPENSES (Itemize)

TOTAL DIRECT COST (Enter on Page 1, Item 5) → 67,750

INDIRECT COST (See Instructions)

DATE OF DHEW AGREEMENT:

_____ % S&W*

52.83 % TDC* Provisional: 12/19/79

☐ WAIVED.

☐ UNDER NEGOTIATION WITH:

*IF THIS IS A SPECIAL RATE (e.g. off-site), SO INDICATE.

1079488

BUDGET ESTIMATE FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)

DESCRIPTION		1ST PERIOD (SAME AS DETAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED *(This application only)*					
			2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS		54,800	58,650	63,050				
CONSULTANT COSTS *(Include fees, travel, etc.)*								
EQUIPMENT		950	0	0				
SUPPLIES		11,000	11,600	12,400				
TRAVEL	DOMESTIC	1,000	1,000	1,200				
	FOREIGN							
PATIENT COSTS								
ALTERATIONS AND RENOVATIONS								
OTHER EXPENSES		0	500	500				
TOTAL DIRECT COSTS		$67,750	$71,750	$77,150				
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD *(Enter on Page 1, Item 4)* →						$ 216,650		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Recurring Increases in Personnel Costs computed at 7%.

Equipment Justification

1st Year - Waterbath Shaker - Precision-Dubnoff - $950 - To be used for in vitro enzymological work which must be carried out a controlled temperature with constant oscillations.

SECTION II – PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Myles C. Cabot	Biochemist	[redacted]
PLACE OF BIRTH (City, State, Country)	**PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)**	**SEX**
[redacted] Massachusetts	U.S.A.	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
[redacted]	[redacted]	[redacted]	[redacted]

HONORS

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Lipid metabolism in membranes	Principal Investigator

RESEARCH SUPPORT *(See instructions)*

None

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)*

November 1978-Present: Scientist I, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee (Biochemist, April 1979)

1976-1978: Damon Runyon-Walter Winchell Postdoc. Fellow in Cancer Research, Med. Hlth. Sci., ORAU, Oak Ridge. TN

1975-1976: Instructor; 1974-1976: Assistantship; 1972-1974, Fellowship--The Hebrew Univ. Jerusalem, Israel.

1970-1972: Graduate Assistantship; 1969-1972, Laboratory Assistant--Western Carolina Univ., Cullowhee, NC

PUBLICATIONS

Cabot, M. C. and Gatt, S. *Lipase activity in rat brain.* Isr. J. Med. Sci. 11, 1194, 1975 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of neutral glycerides by lipases of rat brain microsomes.* Biochim. Biophys. Acta 931, 105-115, 1976.

Cabot, M. C. and Gatt, S. *Substrate specificity of the microsomal acid lipase of rat brain.* Isr. J. Med. Sci. 12, 1368, 1976 (abstract)

Cabot, M. C. and Gatt, S. *Hydrolysis of endogenous diacylglycerol and monoacylglycerol by lipases in rat brain microsomes.* Biochemistry 16, 2330-2334, 1977.

Cabot, M. C. and Gatt, S. *Rat brain microsomal lipase activity.* Adv. Exp. Med. Biol. 101, 101-111, 1978.

1079490

1. (Last, first, middle initial)
CABOT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

Cabot, M. C. and Snyder, F. *The manipulation of fatty acid composition in L-M cell monolayers supplemented with chaulmoogric acid.* Arch. Biochem. Biophys. 190, 838-844. 1978.

Cabot, M. C. and Snyder, F. *Assimilation of unnatural aliphatic moieties into complex lipids of neoplastic cells.* In: XII International Cancer Congress, Buenos Aires, Argentina, Abstracts, Workshops, Vol. 1., 1978, p. 177 (Abstract 18).

Cabot, M. C. and Gatt, S. *The hydrolysis of triacylglycerol and diacylglycerol by a rat brain microsomal lipase with an acidic pH optimum.* Biochim. Biophys. Acta 530, 508-512, 1978.

Cabot, M. C. and Snyder, F. *Manipulation of alkylglycerolipid levels in cultured cells Fatty alcohol versus alkylglycerol supplements.* Biochim. Biophys. Acta (in press).

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

10794 91

E (last, first, middle initial)
ABBOT, Myles C.

[redacted] NUMBER

RESEARCH PLAN

A. SPECIFIC AIMS

12-O-Tetradecanoylphorbol-13-acetate (TPA) applications to mouse skin result in the production of both benign and malignant tumors if the TPA treatment is preceded by a single, subcarcinogenic application of an initiating agent, such as 7,12-dimethylbenz[a]anthracene (Baird and Boutwell, 1971; Hecker, 1971; Van Duuren, 1969). However, it is significant to note that phorbol esters, when used in the absence of tumor initiators induce rather than inhibit differentiation in human leukemia cells (Huberman and Callaham, 1979) and stimulate differentiated functions in human melanoma cells (Huberman et al., 1979). We have recently initiated studies of the alterations in lipid metabolism elicited by treatment of human leukemic cells in culture with the potent tumor promoter, TPA. These studies revealed that TPA has a profound effect on lipid metabolism, in particular phosphatidylcholine.

As phospholipids are an integral component of plasma membranes and intracellular organelles, their possible role in cellular differentiation warrants closer investigation. Furthermore, we have observed that TPA treatment causes alterations in the levels of alkyldiacylglycerols. This class of ether-linked lipids, and their polar counterparts, 1-O-alkyl-2-acylglycerophosphatides, occur at characteristically high levels in cancer cells (Snyder and Wood, 1968, 1969).

Specific goals are to establish what alterations in cellular lipid composition occur during TPA-induced differentiation of human cancer cells, with particular regard to the levels of phosphatidylcholine and membrane-associated O-alkyl and O-alk-1-enyl glycerophosphatides. Once these changes are established, investigations at the cellular and molecular levels regarding the effect of TPA on the enzymes, substrates, and cofactors of phosphatidylcholine metabolism will be carried out. These studies will be initiated using cell cultures to determine rates of incorporation of acyl groups versus polar head groups and glycerol and glucose via de novo synthesis. The experiments will be designed to determine turnover of different components of the molecule during TPA-induced differentiation. In turn, we will utilize cell-free homogenates to assess which enzymes of phosphatidylcholine synthesis are affected during TPA exposure. Those activities that will be investigated include choline kinase, CTP-phosphocholine, cytidylyltransferase, and CDP-choline:1,2-diacylglycerol phosphocholinetransferase. Additional emphasis will be placed on determining the effects of TPA on the intracellular levels of required substrates and cofactors such as CTP, phosphocholine, CDP-choline, choline, and diacylglycerol.

B. SIGNIFICANCE

Berenblum (1941) discovered that a single application of benzpyrene to mouse skin followed by multiple applications of croton oil had the remarkable ability to elicit tumor growth. The carcinogenic principles of croton oil are the 12,13-diesters of the polyfunctional, tetracyclic diterpene parent alcohol phorbol (Hecker, 1966). 12-O-Tetradecanoylphorbol-13-acetate, the most abundant and most active tumor promoting agent of croton oil, has been used widely in the two-stage mouse skin carcinogenesis system (Boutwell, 1978) to study the mechanism of tumor promotion. The use of tissue culture systems has aided greatly in elucidating the biological consequences of TPA exposure. Early work with cultured HeLa-cells has demonstrated the effectiveness of TPA as a thymidine blocking agent, a stimulator of choline incorporation, and an inducer of cell growth (Suss et al., 1972). Recent studies have shown that when tumor promoters are used singly they inhibit spontaneous and induced differentiation

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1019492

in murine erythroleukemia cells (Yamasaki et al., 1977; Fibach et al., 1979; Rovera et al.,1977), mouse neuroblastoma cells (Ishu et al., 1978), 3T3 fibroblasts (Diamond et al., 1977), and avian myoblasts (Cohen et al., 1976). Conversely, studies with human myeloid leukemia cells (Huberman and Callaham, 1979; Rovera et al., 1979) and human melanoma cells (Huberman et al., 1979) have revealed a rather dramatic action of phorbol esters: one of induction rather than inhibition of cellular differentiation.

In addition to the previously discussed dichotomy of events elicited by TPA on cellular differentiation, tumor-promoting phorbol esters significantly alter lipid metabolism. Rohrschneider and Boutwell (1973) and Balmain and Hecker (1974) examined the relationship between TPA-induced tumor formation in mouse epidermis and phospholipid metabolism and found that TPA stimulated the early synthesis of phosphatidylethanolamine and phosphatidylcholine. In bovine lymphocytes TPA causes an enhanced labeling with [methyl-^{3}H]choline of phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin (Wertz and Mueller, 1978). Likewise, HeLa cells incubated with low concentrations of TPA (10^{-9} to 10^{-8} M) show a rapid increase of choline incorporation into cellular phosphatidylcholine (Kinzel et al., 1979). The possibility that TPA changes only the permeability of the plasma membrane for the radioactive precursor was excluded, since the effect of TPA on lipid metabolism was also observed when the cells were preincubated with labeled choline. In addition to the general effect of TPA on phospholipid metabolism, TPA has also been shown to evoke other lipid-associaed alterations in a variety of systems: these include modifications of lipid microviscosity in lymphoblastoid cells (Castagna et al., 1979), inhibition of adipose conversion in 3T3 fibroblasts (Diamond et al., 1977), stimulation of secretion of disaturated phosphaidylcholine from alveolar type II cells (Dobbs and Mason, 1978), enhancement of phospholipase activity and prostaglandin production (Levine and Hassid, 1977; Levine and Ohuchi, 1978), and alterations in ganglioside metabolism in human melanoma cells (Huberman et al., 1979).

Whereas, a number of investigators have shown that TPA supplementation to cell cultures causes myriad alterations in lipid metabolism, the diverse effects of phorbol esters on cellular differentiation provides a morphological marker for correlating with TPA-induced modifications of lipid metabolism. In this regard, a cell system in which markers for differentiation can be followed would provide a unique model for studying lipid-related biochemical events during the differentiation process. Human myeloid leukemia cells (HL-60) are extremely useful for such studies, since a large population of the cells (more than 90%) can be induced to differentiate by phorbol diesters (Huberman and Callaham, 1979). TPA-Induced differentiation in these cells has been characterized by morphological changes, decreased cell division, an increase in the percentage of phagocytizing cells, attachment of the cells to the substratum (Huberman and Callaham, 1979) and stimulation of synthesis and extracellular release of lysozymes (unpublished data). This last finding is of interest in veiw of the fact that TPA can cause lysozyme release from peripheral blood polymorphonuclear leukocytes, but not from alveolar macrophages (Goldstein et al., 1975; Bigger, 1978).

Lipids are important components of cellular membranes and membranes are important targets for phorbol esters (Wenner et al., 1974; Lee and Weinstein, 1978; Sivak et al., 1972). Under conditions of in vitro exposure, TPA has been shown to effectively increase the specific activities of the plasma membrane enzymes Na^+-K^+-ATPase and 5'-nucleotidase; the microsomal enzymes, NADH diaphorase and glucose-6-phosphatase, were not affected (Sivak et al., 1972). However, no

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079493

information is available that defines the effects of in vitro TPA exposure on enzymes of phosphatidylcholine biosynthesis.

As has been shown by numerous investigators, phosphatidylcholine is the crucial lipid affected by TPA. The majority of these studies have dealt with the gross effects of TPA on lipid metabolism. Essentially no information is available at the molecular level regarding the effect of TPA and induced cellular differentiation on the enzymes and cofactors responsible for phosphatidylcholine synthesis.

Phosphatidylcholine, a structural component of animal cells, has been implicated in the modulation of certain enzyme activities (Finean, 1973). It is therefore possible that this membrane constituent plays a role in cell regulation. In this respect, the relationship of phosphatidylcholine to cellular differentiation has received little attention, mainly because of the lack of an appropriate model system wherein modifications of phospholipid metabolism can be correlated with the appearance of differentiation markers. The experimental system described herein provides numerous advantages that will facilitate the investigation of phorbol diesters as modulators of cell differentiation and phospholipid metabolism.

The major pathway for phosphatidylcholine biosynthesis was elucidated by Kennedy and coworkers (1962). A second mode of synthesis involving the methylation of phosphatidylethanolamine by the transfer of methyl groups from S-adenosyl-methionine was described by Bremer and Greenberg (1961). Lands and his colleagues were the first to demonstrate the deacylation-reacylation cycle of phosphatidylcholine synthesis (Lands, 1960; Lands and Merkl, 1963). Although of minor significance, phosphatidylcholine can additionally be synthesized by a base exchange reaction (Van Golde and Van den Bergh, 1977). Of the enzymes involved in phosphatidylcholine biosynthesis, it now appears that the regulated step is catalyzed by the soluble enzyme, CTP-phosphocholine cytidylyltransferase (Sundler et al., 1972; Vance and Paddon, 1978; Vance et al., 1980). The first step in phosphatidylcholine biosynthesis, the conversion of choline to phosphocholine, is catalyzed by choline kinase; the last step in phosphatidylcholine biosynthesis is under the control of CDP-choline:1,2-diacylglycerol phosphocholine transferase and converts diacylglycerol and CDP-choline to phosphatidylcholine. With the evidence that tumor promoters elicit remarkable alterations in the metabolism of phosphatidylcholine in bovine lymphocytes (Wertz and Mueller, 1978) and HeLa cells (Kinzel et al., 1979), there has been little advancement regarding the mechanism responsible for this effect. It has been shown that one of the earliest effects elicited by tumor-promoting phorbol esters is the stimulation of phosphatidylcholine metabolism (Kreibich et al., 1971; Suss et al., 1971, 1972). These results implicate the involvement of membranes and their lipid components in the mechanism of cellular growth and differentiation; phospholipid metabolism has been shown to play an important role in the events leading to the stimulation of growth (Aizawa and Mueller, 1961; Cunningham, 1972; Fex, 1970; Pasternak, 1972; Spooner and Gorski, 1972).

The following points need to be considered in proposing the mechanism by which TPA stimulates phospholipid metabolism. Wertz and Mueller (1978) have shown that the phospholipid response to TPA is insensitive to the inhibition of RNA and protein synthesis. Additionally, these investigators have evidence to indicate that TPA activation of phospholipid metabolism does not require a major redistribution of phospholipid-metabolizing enzymes. Thus, the mechanism of action of phorbol esters may involve a direct effect on the phospholipid synthetic enzymes. With these factors in mind, a thorough investigation of

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079494

N[illegible] (last, first, middle initial)
([illegible]OT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

the phospholipid metabolizing enzymes during TPA-induced cellular differentiation will also lend information regarding the role of phosphatidylcholine in cell growth and differentiation.

C. PRELIMINARY STUDIES

We have utilized HL-60 cells thus far in our preliminary studies to investigate both polar and neutral lipid metabolism in hyperplastic (control) and TPA-differentiating systems. TPA caused an enhanced incorporation of ^{32}P into total cellular phospholipids (Fig. 1). This stimulation was detected within

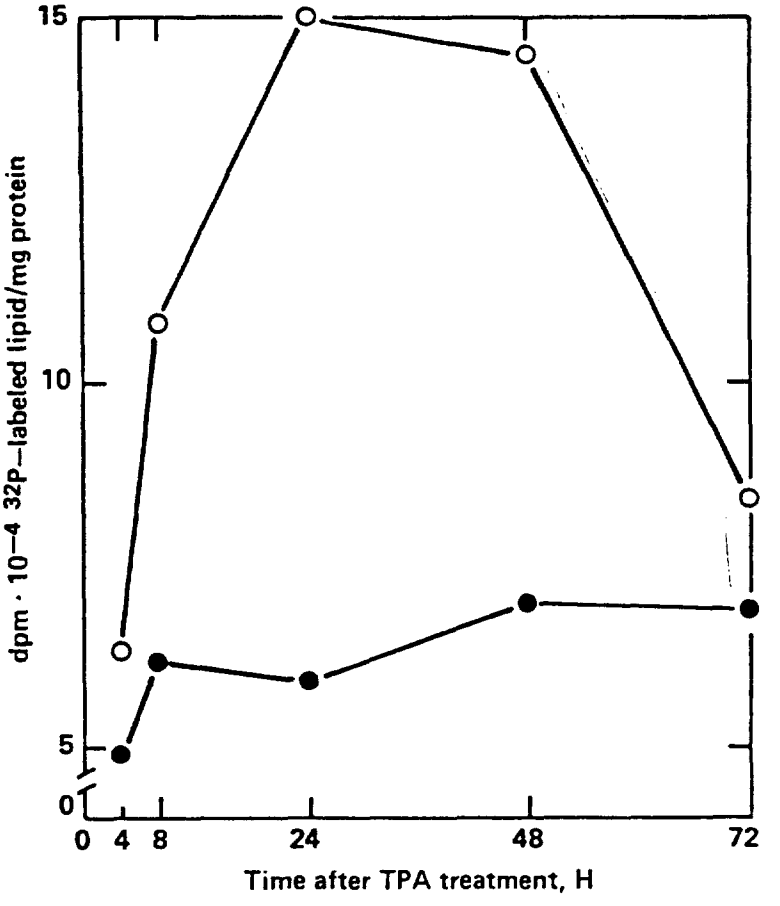

Figure 1. Stimulation of ^{32}P incorporation into phospholipids of HL-60 cells after treatment with TPA. Cells were incubated with TPA (1.7×10^{-10} M) for the times indicated, and ^{32}P (300 μCi/culture) was added to the cultures 4 hr before analysis. TPA and ^{32}P were added simultaneously for a 4-hr incubation. Cells incubated without TPA served as controls. The results are expressed as dpm ^{32}P per mg cell protein; identical results were obtained when the data were expressed as dpm ^{32}P per μg phospholipid Pi. (●——●, untreated; o——o, TPA treated).

4 hr after treatment and preceeded the TPA-induced stimulation of lysozyme synthesis, phagocytosis, and morphological differentiation. Phospholipid fractionation revealed that the metabolic stimulation of ^{32}P incorporation was confined mainly to the choline- and ethanolamine-containing glycerophospholipids; sphingomyelin + lysophosphatidylcholine and phosphatidylinositol + phosphatidylserine were unaffected (Fig. 2). Treatment of the cells with 4-O-methyl-TPA,

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

10194945

(last, first, middle initial)
C...OT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

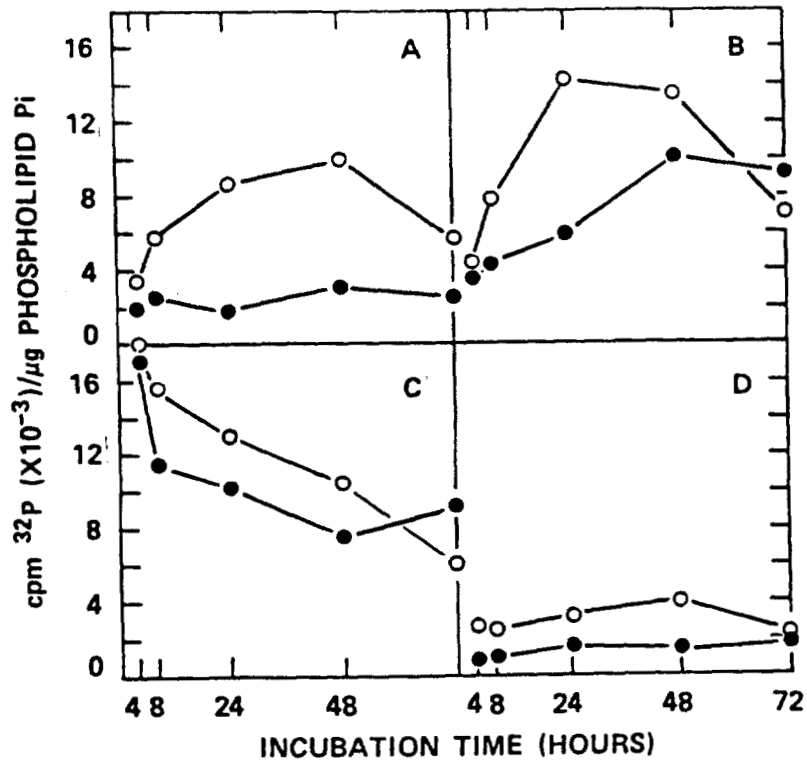

Figure 2. The effect of TPA on ^{32}P incorporation into individual phospholipids of HL-60 cells. Aliquots of total lipid from the experiment described in Figure 1 were separated into phospholipid classes by thin-layer chromatography in chloroform-methanol-acetic acid-water (50:25:8:4, v/v). The lipids were visualized by H_2SO_4 charring, and the individual areas were assayed for phospholipid phosphorus. ^{32}P zonal profile scans of all samples were used to calculate the contribution of the individual labeled phospholipids to the total lipid extract. A: phosphatidylcholine; B: phosphatidylethanolamine; C: phosphatidylinositol + phosphatidylserine; D: sphingomyelin + lysophosphatidylcholine. (●——●, control cells; o——o, TPA-treated cells).

(1.7×10^{-10} M, 24 hr), an analog with poor tumor-promoting activity, failed to elicit any alteration in phospholipid metabolism. Supplementation of growth medium with different amounts of TPA for 24 hr resulted in a dose-related stimulation of ^{32}P incorporation into the cellular phospholipids (Fig. 3).

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

10744 46

NAME (last, first, middle initial)
BOT, Myles C.

SOCIAL SECURITY NUMBER
238-80-9405

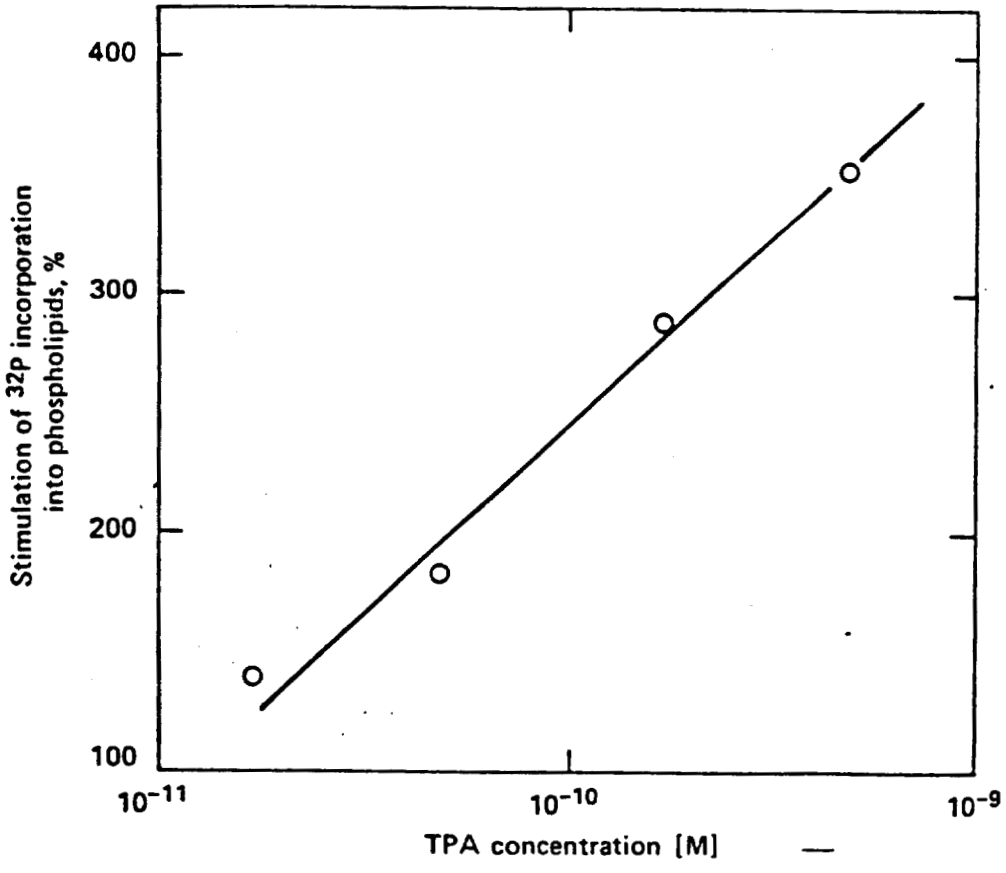

Figure 3. Stimulation of phospholipid metabolism in HL-60 cells after treatment with various concentrations of TPA. Cells were incubated for 24 hr in medium containing TPA. ^{32}P (300 μCi/culture) was added at 20 hr and the lipids were extracted 4 hr later. The amount of lipid ^{32}P incorporated into the untreated cells was designated as 100%.

This dose-response correlates with the susceptibility of the cells to the induction of cell differentiation by TPA. Cells cultivated in the presence of TPA (1.7×10^{-10} M, 24 hr) showed a 3-fold increase over controls in the incorporation of [1,2-^{14}C]choline; these results were identical with the ^{32}P findings.

We have expanded this investigation to compare the metabolism of neutral lipids and phospholipids in control and TPA-exposed cells. The data in Table I

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

L6464 01

N (last, first, middle initial)
C. OT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

TABLE I

The utilization of [^{14}C]acetate for lipid synthesis in control and TPA-supplemented HL-60 leukemia cells

HL-60 cells were plated 1 day before treatment with TPA (1.7×10^{-10} M) and incubated simultaneously with 10 μCi [2-^{14}C]acetate, specific activity 54 μCi/μmol, for 24 hr. The cells were harvested and total lipids extracted and analyzed as detailed in Methods (p.18). Data are based on dpm in lipid per mg cell protein. The lipid classes were resolved by thin-layer chromatography.

Incubation conditions	Label distribution (dpm)			
	Phospholipids	Free Sterols[a]	Free Fatty Acids	Neutral lipids[b]
Control	351,111	19,875	27,796	42,425
TPA-supplemented	268,014	23,467	47,046	75,617

[a]Obtained from the unsaponifiable fraction in total lipid and verified as sterol by thin-layer chromatography.

[b]Neutral lipid refers to the combined triacylglyerol-alkyldiacylglycerol fraction.

demonstrate the differential utilization of [^{14}C]acetate for the synthesis of cellular fatty acids and its incorporation into complex polar and neutral glycerolipids. In differentiating cells versus controls, a 70% increase in the incorporation of acetate into the cellular free fatty acids was observed. Likewise, the utilization of labeled fatty acids for neutral glycerolipid synthesis was markedly enhanced in the TPA-treated cultures (78% over control). Only a small increase of radioactivity in the sterol fraction occurred in treated cells. TPA stimulation of phospholipid metabolism was characterized by an enhanced incorporation of ^{32}P and labeled choline into cellular phospholipids. However, the preliminary evidence seen from the utilization of [1-^{14}C]acetate suggests that the fatty acids synthesized are preferentially incorporated into neutral lipids. The effect of TPA on acyl group turnover in phospholipids has not been investigated. Therefore we can only speculate as to the reason for the reduction of [^{14}C]acetate-phospholipid radioactivity in TPA-supplemented cells. A stimulation of neutral lipid synthesis takes place during cellular differentiation (Table I). In order to determine if the isotope data reflect an actual increase in the net amounts of cellular lipid, total lipids from control and TPA-treated cells were fractionated and quantitated (Table II). On a per mg

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

10794 98

NAME (last, first, middle initial)
ROT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

TABLE II
Lipid composition of control and TPA-treated HL-60 leukemia cells

Cells were incubated in the absence or presene of TPA (1.7×10^{-10} M) for 48 hr. Cells were harvested, washed, and lipids extracted. Amounts of neutral lipids were determined by photodensitometry after resolution on thin-layers of Silica Gel G.

Incubation Conditions	Lipid composition[a]			
	Phospholipid	Sterols	Triacylglycerols	Alkyldiacylglycerols
Control	125	33	42	2.5
TPA-treated	134	36	69	3.9

[a]Values are given as μg lipid per mg protein.

protein basis, the TPA-treated cells showed a 64% increase in triacylglycerols over the controls. Although the ether-containing lipids account for a small amount of the total lipids in HL-60 cells, an increase similar to that observed in the triacylglycerols occurred in the TPA-exposed cells. The amount of the combined wax ester-cholesterol ester fraction in both control and treated cells remained identical. TPA treatment did not alter the amount of cellular sterols, nor was there a significant increase in the amount of total phospholipids. It thus appears from these data and experiments in which the phosphatidylcholine Pi was measured, that TPA does not increase phosphatidylcholine levels in the cells, but rather influences its turnover rate.

Our more recent studies have centered on ether lipid metabolism utilizing labeled fatty alcohols as alkyl group precursors. An enhanced incorporation of hexadecanol into neutral lipids (123% over control) resulted in TPA-treated HL-60 cells. [1-^{14}C]Hexadecanol can contribute to both the synthesis of ether- and ester-linked lipids; measurements showed ether lipid synthesis to be approximately 50% higher in TPA-exposed cells. These data reflect the total labeled ether lipid content in cellular lipids and do not differentiate the proportion of ether-linked phosphoglycerides and neutral alkyldiacylglycerols. Preliminary analyses of the phospholipid fraction (HL-60 cells) show that TPA stimulates plasmalogen production, whereas the alkyl group composition of treated cells is unchanged.

I was trained in the area of lipid biochemistry, and more specifically, lipid enzymology. The pursuit of these disciplines has given me considerable experience in fields of lipid metabolism, membrane biochemistry (membrane-enzyme-substrate interactions, membrane modification), and tissue culture. As a result of my present position, I feel well qualified to pursue the studies on the effect of TPA on ether lipid composition. The facility at the ORAU Medical and Health Sciences Division is well equipped for carrying out the specialized analyses that are required to explore all facets of lipid biochemistry.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1074449

D. METHODS OF PROCEDURE

1. Alterations in cellular lipid composition during TPA-induced differentiation

The modification of phosphatidylcholine metabolism is the major lipid effect elicited during TPA-induced differentiation. However, as we have noted in our preliminary studies, the triacylglycerol and alkyldiacylglycerol content increases in leukemia cells exposed to TPA. In our initial studies we will compare the cellular lipid composition of control and TPA-exposed cells, varying concentration and time of exposure to TPA, to assess the effects of induced differentiation on the levels of phosphatidylcholine, phosphatidylethanolamine, triacylglycerols, and alkyldiacylglycerols. Additionally, cancer cells have been shown to contain elevated amounts of ether-linked lipids. In this respect no information is available regarding the effects of TPA on the alkyl and alk-1-enyl composition in differentiating cells. Here, we will limit our analyses to the phospholipids for two reasons: 1) membranes appear to be the major site of TPA action; 2) any disturbance in phospholipid metabolism would appear to affect the ether lipid pathway. The general protocol for lipid compositional analyses during induced differentiation will involve growing the cells in media containing TPA (1.7×10^{-10} M for most experiments) for periods up to 4 days. The extracted lipids from control and treated cells will be analyzed by thin-layer chromatography and quantitated by photodensitometry.

2. Studies of phosphatidylcholine metabolism using radiolabeled precursors

TPA has been shown to significantly stimulate the incorporatwon of ^{32}P and [^{14}C]choline into phosphatidylcholine. We will extend these studies to investigate the effects of TPA on a) the coupling of choline transport and phosphorylation, b) phosphatidylcholine acyl group metabolism, and c) de novo synthesis of phosphatidylcholine from labeled glycerol and glycerol precursor. Results from these studies will provide preliminary information on which parts of the molecule are effected by TPA and give supportive information for further studies involving the molecular mechanism by which TPA stimulates phosphatidylcholine turnover.

[1-^{14}C]Palmitic and [1-^{14}C]oleic acid will be either added to the growth medium (plus serum, serum-free, or delipidated serum) in 20 µl of absolute ethanol or sonicated in a small amount of medium and added to cultures. In most instances labeled precursors will be entered 24 hr after TPA addition. Water soluble compounds, such as glycerol, glucose, and choline, will be added as solutions made in culture medium. Incubation time in the presence of label will be varied according to the factors being studied. After the appropriate labeling period, cells will be extensively washed in cold saline, isolated by brief centrifugation, and the lipids extracted and analyzed as detailed.

Detailed investigations will be carried out utilizing control and TPA-exposed HL-60 cells to determine if there exist differences between the rate of uptake of [Me-^{3}H]choline in control (neoplastic) and differentiating cell systems. Our initial experiments with ^{32}P and [1,2-^{14}C]choline suggest that TPA modifying permeability is not a factor contributing to the rapid stimulation of phosphatidylcholine labeling. However, detailed investigations will be carried out varying time of exposure to TPA and the concentraion of TPA in the incubation medium to more thoroughly assess the effects of TPA on transport and metabolic fate of the labeled precursor. Transport of labeled choline will be studied by measurement of the disappearance of radioactivity from the cell media. The rate of

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

0019500

disappearance of radioactivity from the cell medium (uptake) will be further checked by washing the cells extensively and sonicating the pellet in water for direct radioactive analysis. A fraction of the cell sonicate will be extracted for lipid analysis and the total lipid extracts analyzed by thin-layer chromatography. In conjunction with these studies, pulse-chase experiments will be undertaken to determine the half-life and metabolic fate of labeled choline products (choline, phosphocholine, CDP-choline, phosphatidylcholine). Briefly, control and TPA-exposed cells will be pulse-labeled with [me-^{3}H]choline for 1 hr, after which time the cells will be washed in fresh medium and replated. At various time intervals following the pulse, the cells will be harvested and the water soluble and organic labeled compounds isolated and analyzed by Dowex AG 1-X10 (OH form), Norit A charcoal and thin-layer chromatographic procedures according to the methods of Vance et al. (1980).

As essentially nothing is known on how TPA affects the acyl group metabolism of phospholipids, investigations of acyl group incorporation and turnover will be done. Initially, experiments will be designed to determine the effects of TPA on the incorporation of labeled palmitic and oleic acids (the major phospholipid fatty acids of HL-60 cells, Cabot, unpublished) into cellular phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. Acyl group turnover will be assessed by labeling a series of control and TPA-treated cultures with palmitic or oleic acid for 1 hr. The cultures will then be replated in fresh media and selected at various time intervals to determine the radiospecific activity in the choline-, ethanolamine-, inositol-, and serine-containing phospholipids.

De novo synthesis of phosphatidylcholine from labeled precursors of the glycerol backbone (glycerol, glucose) will be evaluated in control and TPA-differentiating cells to determine if TPA exposure effects differences in the utilization of these molecules for the synthesis of phosphatidylcholine. Double-label experiments will be conducted by incubating cells with [2,^{3}H]glycerol and [2-^{14}C]glycerol; differences in the ratios of ^{3}H to ^{14}C will be indicative of a shift from either the glycerol phosphate pathway or the dihydroxyacetone-P pathway of phosphatidylcholine synthesis. An isotope effect has been shown to influence the measurement of the involvement of these pathways in the synthesis of phosphatidylcholine (Manning and Brindley, 1972; Rognstad, 1974); however, since the experiments will be carried out on a comparative basis (control versus TPA-exposed systems), information gained will be useful in interpreting the effects of TPA on the utilization of glycerol metabolites for phosphatidylcholine synthesis. The net synthesis of phosphatidylcholine from labeled glucose will also be examined. The confinement of label to the glycerol backbone will be determined by Vitride reduction of the phosphatidylcholine fraction to remove labile esters followed by isolation of labeled glycerol (Cabot and Gatt, 1976). Vitride reduction of alkyl phospholipids produces alkylglycerols that will be cleaved by hydrogen iodide treatment to yield a mixture of hydrocarbons and glycerol. The radioactivity in these products can then be quantitated by differential solvent extraction.

3. In vitro assays of the major phosphatidylcholine biosynthetic enzymes.

Optimal assay conditions for choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32), phosphocholine cytidylyltransferase (CTP:choline phosphate cytidylyltransferase, EC 2.7.7.15), and choline phosphotransferase (CDP-choline:1,2-diacylglycerol phosphocholinetransferase, EC 2.7.8.2) will be established using cell homogenates and subcellular fractions from control HL-60 cells. Once these conditions are established, the activities of the enzymes will be compared in control and TPA-exposed cells.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079501

NAME (last, first, middle initial)
CABOT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

a. Choline kinase. HL-60 cells will be homogenized with a Potter-Elvehjem homogenizer in media containing 0.25 M sucrose, 1.0 mM EDTA, and 2.0 mM dithiothreitol. The homogenate is centrifuged at 100,000g for 60 min and the supernatant will serve as the enzyme source. Choline kinase will be assayed as described by Weinhold and Rethy (1974).

b. Phosphocholine cytidylyltransferase. The microsomal and soluble fractions will be isolated from HL-60 cells and used as enzyme source. Phosphocholine cytidylyltransferase will be assayed by the method of Ansell and Chojnacki (1969) as modified by Weinhold et al., (1973).

c. Cholinephosphotransferase. The cholinephosphotransferase will be assayed by incorporation of phosphoryl-[Me-^{14}C]choline into phosphatidylcholine from CDP-[Me-^{14}C]choline. The procedure described by Weiss et al. (1958) and modified by Vance and Burke (1974) will be used in the assay of this enzyme. The microsomal fraction will serve as the source of enzyme. The product, phosphatidylcholine, can be isolated from the reaction mixture by solvent extraction (Vance and Burke, 1974); however, thin-layer chromatography (Silica Gel HR) will be used to confirm product identification. 1,2-Diacyl-sn-glycerol, a substrate in the reaction, will either be obtained commercially or prepared by phospholipase C treatment (Renkonen, 1966) of choline phosphoglycerides isolated from egg or from rat liver. The isomeric purity will be verified by thin-layer chromatography as described by Cabot and Gatt (1978).

4. Measurement of intracellular cofactors of phosphatidylcholine biosynthesis

As TPA may influence the levels of required cofactors of the phosphatidylcholine biosynthetic pathway, it is important to regard this effect as playing a possible role in the TPA stimulation of phosphatidylcholine metabolism. If TPA causes a decrease in the endogenous amount of one of the choline-containing precursors, this would then result in increased radiospecific activity and cause stimulated incorporation of labeled choline into phosphatidylcholine. The measurement of CTP, phosphocholine, CDP-choline, choline, and diacylglycerol will be determined (Vance et al., 1980) in control and TPA-exposed cells.

5. Specifics of procedure

a) Cell culture

The human promyelocytic HL-60 leukemia cells will be grown in bacterial plastic petri dishes (No. 1007, Falcon) in RPMI-1640 medium plus 20% fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cell numbers will be determined from cell counts after excluding cells stained with trypan blue. Cells will be treated with TPA 24 hr after subculture. In most experiments the concentration of TPA in the culture medium will be in the range of 1×10^{-10} M; however, experiments will be conducted using concentrations ranging from 5×10^{-7}--5×10^{-10} M to assess the concentration effects on lipid metabolism. Explicit details of cell culture procedure, TPA supplementation and evaluation of cell differentiation have been cited elsewhere (Huberman and Callaham, 1979). TPA will be added to culture media in DMSO so that its final concentration does not exceed 0.1%. This amount of DMSO has no effect on cell growth or differentiation. We have shown in our preliminary studies that the stimulation of phosphatidylcholine metabolism preceeds the induction of differentiation markers (Huberman and Callaham, 1979). In view of this, it is important to stress that when setting up the individual experiments, careful consideration must be given to parameters such as time of exposure, cell number, and degree

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079502

of cellular differentiation. Therefore, these variables will be investigated exhaustively for the purpose of comparing and contrasting the degree of differentiation with the modifications observed in phosphatidylcholine metabolism.

b) Lipid isolation and analyses

Lipids will be extracted from control and TPA exposed cells by a modified method of Bligh and Dyer (1959) in which the methanol contains 2% glacial acetic acid. Both neutral and polar lipid classes can be resolved by thin-layer chromatography in a variety of solvent systems (Cabot and Snyder, 1980, see Appendix I). Neutral lipids will be separated on layers on Silica Gel G and phospholipids resolved by thin-layer chromatography on Silica Gel HR. A comparison of R_f values with authentic standards will be used for the identification of individual lipid classes. The more refined separations include triacylglycerols and alkyldiacylglycerols, hexane/diethyl ether/acetic acid (80:20:1); cholesterol esters-wax esters-alkyldiacylglycerols, benzene (100%); and alkylglycerol-alk-1-enyl glycerols, diethyl ether/acetic acid/water (100:0.5:0.5). Quantitative analysis of the thin-layer-resolved lipids is accomplished by H_2SO_4 charring of the chromatoplates at 200°C followed by photodensitometry according to Privett et al. (1965). When radiolabeled lipid precursors are utilized in cell culture systems, their distribution in the lipid extract will be determined by zonal profile scanning of the chromatogram (Snyder and Kimble, 1965). The alkyl group content of ether-containing phospholipids will be assessed by Vitride reduction to cleave labile esters followed by resolution of the products, alkylglycerols and alk-1-enylglycerols, on thin-layer plates in a solvent system containing diethyl ether/acetic acid/water (100:0.5:0.5).

An alternative system for plasmalogen identification will be employed to differentiate between choline and ethanolamine plasmalogens (Blank et al., 1973). For phospholipid phosphorus determinations, the lipids will be separated on thin-layer plates (Silica Gel HR) in a system containing chloroform/methanol/acetic acid/water (50:25:8:4, v/v) and visualized by H_2SO_4 charring. The spots are then scraped into test tubes for direct analysis of phosphorus (Rouser et al., 1966).

Changes in the acyl group composition of phosphatidylcholine may indicate in control versus TPA-exposed cells altered physical properties of the membranes. To investigate the effects of TPA on acyl group composition, the phosphatidylcholine fraction from control and treated cells will be isolated by preparative thin-layer chromatography in a solvent system containing chloroform/methanol/acetic acid/water (50:25:8:4). Methyl esters will be prepared by refluxing the phosphatidylcholine in methanol containing 2% H_2SO_4 at 100°C (Ways et al., 1963) and analyzed by gas-liquid chromatography according to the methods described by Blank and Snyder (1970) and Blank et al. (1976). Additionlly, differences in the positional specificity of the fatty acids will be determined by gas-liquid chromatography after treatment of phosphatidylcholine with phospholipase A_2 (Cabot and Snyder, 1978).

6. Priorities

The priorities of our proposed work will be essentially according to the order outlined (pp. 15-18). However, preliminary data will be obtained through lipid compositional studies of cells after specific treatments, and these results will direct future project decisions so that major compositional changes can be investigated from the point of view of establishing the underlying mechanism for the change.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1019503

NAME (last, first, middle initial)
CABOT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

We have a number of collaborative ties with scientists outside our group: cell biologists and toxicologists (Dr. Eliezer Huberman's group, Oak Ridge National Laboratory), organic chemists (Dr. Claude Piantadosi's group at the University of North Carolina, Chapel Hill), and mass spectroscopists at the Oak Ridge National Laboratory (Dr. William Rainey). These scientists could be helpful in solving some of the specialized problems that might arise (cell differentiation characterization, synthesis, and identification of compounds).

Consultant:

Dr. Fred Snyder of our Division has agreed to serve as a consultant in this research project. A copy of his curriculum viate follows (Appendix II).

Timetable

First year: We will concentrate our efforts on determining the cellular lipid compositional changes that occur in response to TPA-induced differentiation. Close scrutiny will be given regarding the influence of concentration and time of exposure to TPA and how this effects the triacylglycerol, alkyldiacylglycerol, phosphatidylcholine, phosphatidylethanolamine, and alkyl group content of experimental cells. Studies will be initiated using labeled compounds to assess the effects of TPA on the coupling of choline transport and phosphorylation, phosphatidylcholine acyl group metabolism, and de novo synthesis of phosphatidylcholine. We will recruit a postdoctoral fellow and technician to begin the second year.

Second year: Studies of phosphatidylcholine synthesis will be completed. The optimal conditions for assaying choline kinase, phosphocholine cycidylyltransferase and cholinephosphotransferase will be established and experiments initiated to determine the effects of TPA-induced differentiation on the activity of these anabolic enzymes.

Third year: Studies on the activities of the phosphatidylcholine biosynthetic enzymes will continue. Simultaneously, experiments will be conducted to measure the effects TPA exerts on the cellular cofactors and substrates required for phosphatidylcholine synthesis.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1019504

REFERENCES

Aizawa, Y. and Mueller, G. C. (1961) J. Biol. Chem. 236, 381
Ansell, G. B. and Chojnacki, T. (1969) Methods Enzymol. 14, 121
Baird, W. M. and Boutwell, R. K. (1971) Cancer Res. 31, 1074
Balmain, A. and Hecker, E. (1974) Biochim. Biophys. Acta 362, 457
Berenblum, I. (1941) Cancer Res. 1, 1941
Blank, M. L. and Snyder, F (1970) Lipids 5, 337
Blank, M. L., Wykle, R. L. and Snyder, F. (1973) Biochim. Biophys. Acta 316, 28
Blank, M. L., Lee, T-c., Piantadosi, C., Ishaq, K. S., and Snyder, F. (1976) Arch. Biochem. Biophys. 177, 317
Bigger, W. D. (1978) Infect. Immun. 21, 669
Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911
Boutwell, R.. L. (1978) In: Mechanisms of Tumor Promotion and Carcinogenesis (Slaga, T. J., Sivak, A. J., and Boutwell, R. L., eds.), Raven Press, New York, p. 49
Bremer, J. and Greenberg, D. M. (1961) Biochim. Biophys. Acta 46, 205
Cabot, M. C. and Gatt, S. (1976) Biochim. Biophys. Acta 431, 105
Cabot, M. C. and Gatt, S. (1978) Biochim. Biophys. Acta 530, 508
Cabot, M. C. and Snyder, F. (1978) Arch. Biochem. Biophys. 190, 838
Cabot, M. C. and Snyder, F. (1980) Biochim. Biophys. Acta (in press)
Castagna, M., Rochette-Egly, C., Rosenfeld, C., and Mishal, Z. (1979) FEBS Lett. 100, 62
Cohen, R., Pacific, M., Rubenstein, N., Biehl, J., and Holtzer, M. (1976) Nature (Lond.) 269, 1232
Cunningham, D. D. (1972) J. Biol. Chem. 247, 2464
Diamond, L., O'Brien, T. G., and Rovera, G. (1977) Nature (Lond.) 269, 247
Dobbs, L. and Mason, R. J. (1978) Am. Rev. Respir. Dis. 118, 705
Fex, G. (1970) Biochem. J. 119, 743
Fibach, E., Gambari, R., Shaw, P. A., Maniatis, G., Ruben, R. C., Sassa, S., Rifkind, R. A., and Marks, P. A. (1979) Proc. Natl. Acad. Sci., USA 76, 1906
Finean, J. B. (1973) In: Form and Function of Phospholipids (Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C., eds.), Elsevir Scientific Publishing Co., Amsterdam, p. 171
Goldstein,I. M., Hoffstein, S. T., and Weissman, G. (1975) J. Cell Biol. 66, 647
Hecker, E. (1966) Proc. IX Int. Cancer Congr., Tokyo (Abstracts, p. 17)
Hecker, E. (1971) Methods Cancer Res. 6, 439
Huberman, E. and Callaham, M. F. (1979) Proc. Natl. Acad. Sci. USA 76, 1293
Huberman, E., Heckman, C., and Langenback, R. (1979) Cancer Res. 39, 218
Ishu, D. N., Fibach, E., Yamasaki, H., and Weinstein, I. B. (1978) Science, 200, 556
Kennedy, E. P. (1962) Harvey Lect. 57, 143
Kinzel, V., Kreibich, G., Hecker, E., and Suss, R. (1979) Cancer Res. 39, 2743
Kreibich, G., Hecker, E., Suss, R., and Kinzel, V. (1971) Naturwissenschaften 58, 323
Lands, W. E. M. (1960) J. Biol. Chem. 235, 2233
Lands, W. E. M. and Merkl, I. (1963) J. Biol. Chem. 238, 898
Lee, L-S. and Weinstein, I. B. (1978) J. Environ. Pathol. Toxicol. 1, 627
Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477
Levine, L. and Ohuchi, K. (1978) Nature (Lond.) 276, 274
Manning, R. and Brindley, D. N. (1972) Biochem. J. 130, 1003
Pasternak, C. A. (1972) J. Cell Biol. 53, 231
Privett, O. S., Blank, M. L., Codding, D. W., and Nickell, E. E. (1965) J. Am. Oil Chem. Soc. 42, 381
Renkonen, O. (1966) Biochim. Biophys. Acta 125, 288

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1019505

Rognstad, R., Clark, D. G., and Katz, J. (1974) Biochem. J. 140, 249
Rohrschneider, L. R. and Boutwell, R. K. (1973) Cancer Res. 33, 1945
Rouser, G., Siakotos, A. N., and Fleischer, S. (1966) Lipids 1, 85
Rovera, G., O'Brien, T. G., and Diamond, L. (1977) Proc. Natl. Acad. Sci. USA 74, 2894
Rovera, G., O'Brien, T. G., and Diamond, L. (1979) Science 204, 868
Sivak, A., Mossman, B. T., and Van Duuren, B. L. (1972) Biochem. Biophys. Res. Commun. 46, 605
Spooner, P. M. and Gorski, J. (1972) Endocrinology 91, 1273
Snyder and Kimble, H. (1965) Anal. Biochem. 11, 510
Snyder, F. and Wood, R. (1968) Cancer Res. 28, 972
Snyder, F. and Wood, R. (1969) Cancer Res. 29, 251
Sundler, R., Arvidson, G., and Akesson, B. (1972) Biochim. Biophys. Acta 280, 559
Suss, R., Kinzel, V., and Kreibich, G. (1971) Experientia 27, 46
Suss, R., Kreibich, G., and Kinel, V. (1972) Eur. J. Cancer 8, 299
Vance, D. E. and Burke, D. C. (1974) Eur. J. Biochem. 43, 327
Vance, D. E. and Paddon, H. B. (1978) Proc. Can. Fed. Biol. Soc. 21, 181
Vance, D. E., Trip, E. M., and Paddon, H. B. (1980) J. Biol. Chem. (in press)
Van Duuren, B. L. (1969) Prog. Exp. Tumor Res. 11, 31
Van Golde, L. M. G. and Van den Bergh, S. G. (1977) In: Lipid Metabolism in Mammals, Vol. 1, (F. Snyder, ed.), Plenum Press, New York, p. 35
Ways, P. Reed, C. F., and Hannahan, D. J. (1963) J. Clin. Invest. 42, 1248
Weinhold, P. A., Skinner, R. S., and Sanders, R. D. (1973) Biochim. Biophys. Acta 326, 43
Weinhold, P. A. and Rethy, V. B. (1974) Biochemitry 13, 5135
Weiss, S. B., Smith, S. W., and Kennedy, E. P. (1958) J. Biol. Chem. 231, 53
Wenner, C. E., Hackney, J., Kimelberg, H. K., and Mayhew, E. (1974) Cancer Res. 34, 1731
Wertz, P. W. and Mueller, G. C. (1978) Cancer Res. 38, 2900
Yamasaki, H., Fibach, E., Nudel, U., Weinstein, I. B., Rifkind, R. A., and Marks, P. A. (1977) Proc. Natl. Acad. Sci. USA, 74, 3451

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079506

NAME (last, first, middle initial)
DT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

E. FACILITIES AVAILABLE

Our laboratories are well equipped to conduct the experiments outlined in this proposal. We have recently added a new tissue culture facility containing both forced draft and water-jacketed incubators equipped with automatic CO_2 control, laminar flow hoods, a self-contained biohazard hood, coulter counter, and liquid nitrogen refrigerator. For lipid analyses we have a complete line of thin-layer chromatographic equipment, including a thin-layer zonal scraper, a spark chamber for chromatographic detection of ^3H and ^{14}C, and a photodensitometer designed for quantitative analysis. We also have the following equipment: preparative and analytical centrifuges with conventional rotors, an analytical Model E ultracentrifuge, liquid scintillation spectrometers, gas-liquid chromatographs with a variety of column packings, an automatic freeze dryer, Beckman DU and Acta C-III spectrometer, equipment for isolating proteins (electrophoresis apparatus, fraction collectors, columns), and a Cahn electrobalance (Model 25). In other sections of our Division we have access to high pressure liquid chromatographs and electron microscope service.

'USE OF DOE FACILITIES AND DOE CONTRACT REQUIREMENTS'

This research grant application includes a segment of activity that would be performed in facilities of the U. S. Department of Energy and governed by an existing contract between Oak Ridge Associated Universities (ORAU) and the DOE. The DOE has reviewed this proposal and has concurred in ORAU conducting the described work in the DOE facilities made available for biomedical research, subject to payment to the DOE by ORAU from NIH funds of the applicable direct and indirect cost of the work (not including any charge for the use of DOE facilities) as determined by the provisions of the DOE's contract with ORAU.

It is belived that in large measure the requirements of the DOE contract parallel conditions that NIH ordinarily applies to its grants. In the event of differences between NIH grant terms and the DOE contract terms, ORAU is agreeable to meeting both to the extent that they are not in conflict, and to applying those most favorable to the United States Government where this is involved. If NIH is aware of problems that such an approach would produce or suggest, ORAU upon receipt of such advice would refer the matter to the DOE for direct resolution with NIH.

By way of general information, ORAU's contract with the DOE is a cost-type contract financed under a Government-fund account. The specific contract work is formulated in cooperation with the DOE and authorized within general guidelines in the contract. Contract terms include DOE responsibility for Government ownership and control of inventions, data, and other research products. Ownership of all equipment and facilities acquired by ORAU with DOE funds is vested in the U. S. Government at the time of acquisition. The contract also contains all the terms generally common to Government contracts of the type under which ORAU conducts research operations in Government-owned facilities.

F. Collaborative Arrangements: None

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079507

Privileged Communication

NA (last, first, middle initial)
CABOT, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

Continuation page

G. Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

Principal Investigator

Feb 26, 1980

Date

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

10179508

JAN 1 8 1980

W. W. Burr, Director, Office of Health and Environmental Research, EV-30, HQ (Mail Station E-201, Germantown)

ORAU DRAFT GRANT APPLICATION ENTITLED "ETHER LIPID METABOLISM IN CELL TRANSFORMATION" (DOE 20-78-80)

Attached for your review and approval is a new grant application to NIH prepared by ORAU. The subject project, if approved, will be under the direction of Dr. Myles Cabot. Total direct funding of $233,900 is requested for the three-year proposed project period beginning December 1, 1980, through November 30, 1983. Direct funding requested for the first year is $86,000.

DOE support will consist of the use of equipment and facilities assigned to the ORAU Medical and Health Sciences Division without charge. Please advise whether you approve joint DOE support for this project based on DOE's programmatic interest.

ORIGINAL SIGNED BY
K. M. HAYTHORN

K. M. Haythorn, Director
Energy Programs and Support Division

ER-13:REB

Attachment: NA
Grant Application
w/ORAU ltr 1-14-80

cc w/attachment:
John Maddox, EV-513, HQ (MS E-201, Germantown)

cc w/o attachment:
W. P. Snyder, CC-10, ORO

CONCURRENCES	
RTG. SYMBOL	ER-13
INITIALS/SIG.	Benson, DJ
DATE	1-22
RTG. SYMBOL	ER-13
INITIALS/SIG.	WPB
DATE	1-23-8
RTG. SYMBOL	ER-11
INITIALS/SIG.	Haythorn
DATE	1-28-80
RTG. SYMBOL	
INITIALS/SIG.	
DATE	
RTG. SYMBOL	
INITIALS/SIG.	
DATE	
RTG. SYMBOL	
INITIALS/SIG.	
DATE	
RTG. SYMBOL	
INITIALS/SIG.	
DATE	
RTG. SYMBOL	
INITIALS/SIG.	
DATE	

A-211

20-78-E

DOE FORM AD-9 (12-77)

OFFICIAL FILE COPY

1079509

Oak Ridge
Associated
Universities

January 14, 1980

Mr. Kenneth M. Haythorn
Energy Programs and Support Division
Department of Energy
Oak Ridge, Tennessee 37830

Subject: DRAFT GRANT APPLICATION ENTITLED "ETHER LIPID METABOLISM IN CELL TRANSFORMATION"

Dear Mr. Haythorn:

Enclosed are four copies of a new grant application entitled "Ether Lipid Metabolism in Cell Transformation". This project will be under the direction of Dr. Myles C. Cabot and, if approved, will be carried out under procedures and policies already established between ORAU and the DOE for work in facilities owned by DOE. Should any questions arise during your review of this proposal please do not hesitate to call Dr. Cabot at extension 6-3122.

Formal copies of this application should be forwarded to NIH no later than February 25, 1980.

Sincerely,

Philip L. Johnson

Philip L. Johnson
Executive Director

RYAN:br

Enclosures

20-78-80

1079510

Form Approved
O.M.B. 68-R0249

SECTION I

DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

DRAFT

GRANT APPLICATION

LEAVE BLANK		
TYPE	PROGRAM	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL (Month, Year)		DATE RECEIVED

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR (Items 1 through 7 and 15A)

1. TITLE OF PROPOSAL (Do not exceed 53 typewriter spaces)
ETHER LIPID METABOLISM IN CELL TRANSFORMATION

2. PRINCIPAL INVESTIGATOR

2A. NAME (Last, First, Initial)
Cabot, Myles C.

2B. TITLE OF POSITION
Biochemist

2C. MAILING ADDRESS (Street, City, State, Zip Code)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

2D. DEGREE
Ph.D.

2E. SOCIAL SECURITY NO.
[redacted]

2F. TELEPHONE DATA — Area Code: 615 — TELEPHONE NUMBER AND EXTENSION: 576-3122

2G. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT (See Instructions)
Biological Chemistry

2H. MAJOR SUBDIVISION (See Instructions)
Medical and Health Sciences Division

3. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
FROM: December 1, 1980 — THROUGH: November 30, 1983

4. TOTAL DIRECT COSTS REQUESTED FOR PERIOD IN ITEM 3
$233,900

5. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH PERIOD
$86,000

6. PERFORMANCE SITE(S) (See Instructions)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

Third Congressional District

7. Research Involving Human Subjects (See Instructions)
A. [X] NO B. [ ] YES Approved: _____ Date
C. [ ] YES – Pending Review

8. Inventions (Renewal Applicants Only - See Instructions)
A. [X] NO B. [ ] YES – Not previously reported
C. [ ] YES – Previously reported

TO BE COMPLETED BY RESPONSIBLE ADMINISTRATIVE AUTHORITY (Items 8 through 13 and 15B)

9. APPLICANT ORGANIZATION(S) (See Instructions)
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

(IRS No. 62-0476816)

Third Congressional District

10. NAME, TITLE, AND TELEPHONE NUMBER OF OFFICIAL(S) SIGNING FOR APPLICANT ORGANIZATION(S)
Philip L. Johnson
Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830
Telephone Number(s) 615 576-3300

11. TYPE OF ORGANIZATION (Check applicable item)
[ ] FEDERAL [ ] STATE [ ] LOCAL [X] OTHER (Specify)
Not for profit, private institution

12. NAME, TITLE, ADDRESS, AND TELEPHONE NUMBER OF OFFICIAL IN BUSINESS OFFICE WHO SHOULD ALSO BE NOTIFIED IF AN AWARD IS MADE
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830
Telephone Number 615 576-3056

13. IDENTIFY ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT PURPOSES (See Instructions)
20 Other (Medical and Health Sciences Division)

14. ENTITY NUMBER (Formerly PHS Account Number)
1620476816A1

15. CERTIFICATION AND ACCEPTANCE. We, the undersigned, certify that the statements herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with Public Health Service terms and conditions in effect at the time of the award.

SIGNATURES (Signatures required on original copy only. Use ink, "Per" signatures not acceptable)	A. SIGNATURE OF PERSON NAMED IN ITEM 2A	DATE
	B. SIGNATURE(S) OF PERSON(S) NAMED IN ITEM 10	DATE

PHS-398 (Formerly NIH-398)
Rev. 1-73

Use prescribed by NIH Manual Issuances 4201, 4306, 5005, and 5201.

"THIS APPLICATION DOES NOT INVOLVE RECOMBINANT DNA."

1079511

SECTION 1

DEPAR' NT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

RESEARCH OBJECTIVES

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION

Oak Ridge Associated Universities, P. O. Box 117,
Oak Ridge, Tennessee 37830

NAME, SOCIAL SECURITY NUMBER, OFFICIAL TITLE, AND DEPARTMENT OF ALL PROFESSIONAL PERSONNEL ENGAGED ON PROJECT, BEGINNING WITH PRINCIPAL INVESTIGATOR

Myles C. Cabot [redacted] Principal Investigator

Linda S. Borman [redacted] Cell Biologist

TITLE OF PROJECT

ETHER LIPID METABOLISM IN CELL TRANSFORMATION

USE THIS SPACE TO ABSTRACT YOUR PROPOSED RESEARCH. OUTLINE OBJECTIVES AND METHODS. UNDERSCORE THE KEY WORDS (NOT TO EXCEED 10) IN YOUR ABSTRACT.

The occurrence of elevated levels of ether-linked lipids in neoplastic tissues has been well documented. Although the metabolic pathways of ether lipid metabolism in cancer cells have been elucidated, investigation of the mechanisms responsible for the expression of these lipid changes has been hampered due to the lack of a suitable biological model wherein ether lipid stasis could be followed during the process of cellular differentiation. By utilizing tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate, which induce terminal differentiation in human leukemia and melanoma cells, and tumor initiator-promoter treatment of mouse embryo C3H/10T1/2 cells to elicit two-stage chemical oncogenesis, we will, for the first time, be able to correlate the dynamics of ether lipid metabolism with cellular differentiation. Total lipids isolated from cells during various stages of TPA-induced differentiation, and oncogenic transformation will be rigorously analyzed and the activities of the key enzymes of ether lipid metabolism, *O*-alkyl cleavage enzyme, alkyl synthase, acyl-CoA reductase, and fatty alcohol:NAD^+ oxidoreductase will be characterized in vitro. Other factors contributing to the regulation of ether lipid metabolism will additionally be investigated: cellular utilization of glucose in supplying the glycerol backbone of the alkyl lipids, analysis of cellular DHAP levels, and synthesis of alkyl-containing neutral lipids versus polar alkylglycerophosphatides. The ultimate objective is to understand what factors are responsible for the enrichment of ether-linked lipids in cancer cells which could be later employed as indicators of preneoplastic change.

LEAVE BLANK

1079512

REMOVE AND USE FOR DRAFT COPY

DETAILED BUDGET F FIRST 12-MONTH PERIOD

FROM 12/ 1/80 THROUGH 11/30/81

DESCRIPTION (Itemize)		TIME OR EFFORT %/HRS.	AMOUNT REQUESTED (Omit cents)		
PERSONNEL					
NAME	TITLE OF POSITION		SALARY	FRINGE BENEFITS	TOTAL
Cabot, Myles C. Ph.D.	PRINCIPAL INVESTIGATOR	50	12,120	2,365	14,485
Borman, Linda Ph.D.	Cell Biologist	15	3,580	715	4,295
Welsh, Clement J.	Biochemist	70	10,730	2,415	13,145
TO BE NAMED	Postdoctoral	100	13,000	1,300	14,300
TO BE NAMED	Research Technician	50	7,000	1,575	8,575
			46,430	8,370	54,800

CONSULTANT COSTS

EQUIPMENT
- Waterbath Shaker $950
- Coulter Counter 3,850
- Spectrodensitometer 14,400

19,200

SUPPLIES
- Tissue Culture Ware 2,000
- Cell Culture Media & Serum 3,500
- Chemical 1,500
- Radiolabeled Compounds 4,000

11,000

TRAVEL
- DOMESTIC 1,000
- FOREIGN

PATIENT COSTS (See instructions)

ALTERATIONS AND RENOVATIONS

OTHER EXPENSES (Itemize)

TOTAL DIRECT COST (Enter on Page 1, Item 5) → 86,000

INDIRECT COST (See Instructions)

DATE OF DHEW AGREEMENT:

_____ % S&W*

53.83 % TDC* Provisional: 12/19/79

☐ WAIVED

☐ UNDER NEGOTIATION WITH:

*IF THIS IS A SPECIAL RATE (e.g. off-site), SO INDICATE.

1079513

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED FROM PUBLIC HEALTH SERVICE
DIRECT COSTS ONLY (Omit Cents)

DESCRIPTION		1ST PERIOD (SAME AS DETAILED BUDGET)	ADDITIONAL YEARS SUPPORT REQUESTED (This application only) 2ND YEAR	3RD YEAR	4TH YEAR	5TH YEAR	6TH YEAR	7TH YEAR
PERSONNEL COSTS		54,800	58,650	63,050				
CONSULTANT COSTS (Include fees, travel, etc.)								
EQUIPMENT		19,200	0	0				
SUPPLIES		11,000	11,600	12,400				
TRAVEL	DOMESTIC	1,000	1,000	1,200				
	FOREIGN							
PATIENT COSTS								
ALTERATIONS AND RENOVATIONS								
OTHER EXPENSES								
TOTAL DIRECT COSTS		86,000	71,250	76,650				
TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Enter on Page 1, Item 4) →						$ 233,900		

REMARKS: *Justify all costs for the first year for which the need may not be obvious. For future years, justify equipment costs, as well as any significant increases in any other category. If a recurring annual increase in personnel costs is requested, give percentage. (Use continuation page if needed.)*

Recurring Increases in Personnel Costs computed at 7%.

Equipment Justification

1. Waterbath Shaker - Precision-Dubnoff - $950- To be used for in vitro enzymological work which must be carried out a controlled temperature with constant oscillations.
2. Coulter Counter - model 2F - $3,850 - To be used in cell counting. When cell cultures are manipulated by the action of added agents, an accurate determination of cell numbers will provide essential information on growth rates.
3. Spectrodensitometer - Krotos Schoeffel - $14,400 - (for thin-layer chromatography) - Much of the work will invole assessment of quantitative and qualitative changes in cellular lipids. Total lipid mixtures are routinely separated by thin-layer chromatography. This instrument provides a means to measure directly the quantity of each componentresolved on the chromatogram. Our present densitometer is outdated, subject to heavy use, and due to breakdown of several components, is operating short of its total capabilities.

REMOVE AND USE FOR DRAFT COPY

1079514

RESEARCH PLAN

A. Specific Aims. We have been investigating alterations in lipid metabolism elicited by treatment of human leukemic cells in culture with the potent tumor promotor, 12-O-tetradecanoylphorbol-13-acetate (TPA). These studies revealed that TPA has a profound effect on neutral lipid metabolism, in particular the triacylglycerols and alkyldiacylglycerols. The latter occur at characteristically high levels in cancer cells (Snyder and Wood, 1968, 1969; Wood and Snyder, 1967); however their role in the neoplastic process remains obscure. Although much is known about the metabolism of alkyl lipids (Wykle and Snyder, 1976), a cell culture system wherein the levels of ether-linked lipids can be modified via the action of an outside agent has not yet been developed. It is significant to note that phorbol diesters, when used in the absence of tumor initiators induce rather than inhibit terminal differentiation in human leukemia cells (Huberman and Callahan, 1979) and stimulate differentiated functions in human melanoma cells (Huberman et al., 1979). On the other hand, oncogenic transformation can be evoked in normal cultured cells by sequential treatment with a tumor initiator and promoter (Mondal et al., 1976). These systems offer the unique advantage for investigating the modulation of ether-linked lipids during both the normal to neoplastic cell transformation and the neoplastic to terminally differentiated state. Because ether-linked lipids are closely aligned with neoplastic growth, it is of interest to ascertain at what point during cell transformation the first alterations in ether-lipid synthesis can be detected and moreover what mechanisms are responsible for the expression of these changes.

We will employ the following systems to investigate the link between ether lipids and neoplasia: (a) cancer cell cultures in which differentiation occurs either spontaneously or is induced by the presence of phorbol diesters in the growth medium; (b) cancer cell cultures which are resistant to TPA-induced differentiation; (c) cultures of normal cells which undergo oncogenic transformation via initiator-promoter treatment. Use of cell culture systems manipulated in the manner described

1019515

the enzyme catalyzing the synthesis of plasmalogens; assess the influence of in vitro exposure to TPA on the microsomal enzymes of ether lipid metabolism.

1079516

B. Significance.

Berenblum (1941) discovered that a single application of benzpyrene to mouse skin followed by multiple applications of croton oil had the remarkable ability to elicit tumor growth. The carcinogenic principles of croton oil are the 12,13-di-esters of the polyfunctional, tetracyclic diterpene parent alcohol phorbol (Hecker, 1966). 12-O-Tetradecanoylphorbol-13-acetate, the most abundant and most active tumor promoting agent of croton oil, has been used widely in the two-stage mouse skin carcinogenesis system (Boutwell, 1978) to study the mechanism of tumor promotion. The use of tissue culture systems has aided greatly in elucidating the biological consequences of TPA exposure; early work with cultured HeLa-cells has demonstrated the effectiveness of TPA as a thymidine blocking agent, a stimulator of choline incorporation, and an inducer of cell growth (Süss et al., 1972). Recent studies have shown that when tumor promoters are used singly they inhibit spontaneous and induced differentiation in murine erythroleukemia cells (Yamasaki et al., 1977; Fibach et al., 1979; Rovera et al., 1977), mouse neuroblastoma cells (Ishü et al., 1978), 3T3 fibroblasts (Diamond et al., 1977), and avian myoblasts (Cohen et al., 1976). Conversely, studies with human myeloid leukemia cells (Huberman and Callaham, 1979; Rovera et al., 1979) and human melanoma cells (Huberman et al., 1979) have revealed a rather dramatic action of phorbol esters: one of induction rather than inhibition of cellular differentiation. In addition to this dichotomy of events elicited by TPA on cellular differentiation, tumor-promoting phorbol esters significantly alter lipid metabolism.

The experiments of Rohrschneider and Boutwell (1973) and Balmain et al. (1974) examined the relationship between TPA-induced tumor formation in mouse epidermis and phospholipid metabolism and found that TPA stimulated the early synthesis of phosphatidylethanolamine and phosphatidylcholine.

1079517

In bovine lymphocytes TPA causes an enhanced labeling with [methyl-^{3}H]-choline of phosphatidylcholine, lysophosphatidylcholine and sphingomyelin (Wertz and Mueller, 1978). Likewise, HeLa cells incubated with low concentrations of TPA (10^{-9} to 10^{-8} M) show a rapid increase of choline incorporation into cellular phosphatidylcholine (Kinzel et al., 1979). The possibility that TPA changes only the permeability for the radioactive precursor was excluded, as the effect of TPA on lipid metabolism was also observed when the cells were preincubated with labeled choline. In addition to the general effect of TPA on phospholipid metabolism, TPA has also been shown to evoke other lipid-associated alterations in a variety of systems: these include modifications of lipid microviscosity in lymphoblastoid cells (Castagna et al., 1979), inhibition of adipose conversion in 3T3 fibroblasts (Diamond et al., 1977), stimulation of secretion of disaturated phosphatidylcholine from alveolar type II cells (Dobbs and Mason, 1978), enhancement of phospholipase activity and prostaglandin production (Levine and Hassid, 1977; Levine and Ohuchi, 1978), and alterations in ganglioside metabolism in human melanoma cells (Huberman et al., 1979).

Whereas a number of investigators have shown that TPA supplementation to cell cultures causes myriad alterations in lipid metabolism, the diverse effects of phorbol esters on cellular differentiation lends an added parameter when defining TPA-induced modifications of lipid metabolism. In this regard, a cell system in which markers for differentiation can be followed would provide a rather unique model for studying lipid-related biochemical events during the differentiation process. Human myeloid leukemia cells (HL-60) are extremely useful for such studies since a large population of the cells (more than 90%) can be induced to differentiate by phorbol diesters (Huberman and Callaham, 1979). TPA-induced differentiation in these cells has been

1079518

characterized by morphological changes, decreased cell division, an increase in the percentage of phagocytizing cells, attachment of the cells to the substratum (Huberman and Callaham, 1979) and stimulation of synthesis and extracellular release of lysozyme (unpublished data). This last finding is of interest in view of the fact that TPA can cause lysozyme release from peripheral blood polymorphonuclear leukocytes, but not from alveolar macrophages (Goldstein et al., 1975; Bigger, 1978). A system of like versatility has been discribed by Huberman and collaborators (1979) in which human HO melanoma cell differentiation is stimulated by TPA. These differentiated functions include increased melanin production and formation of dendrite-like structures; alterations in ganglioside metabolism were also demonstrated.

Tissues and cells display characteristic lipid compositions which in many cases are quite specific. Brain contains high amounts of sphingolipids, long-chain hydroxy-substituted, and long-chain odd and even fatty acids (behenic, lignoceric, nervonic, cerebronic); adipocytes are high in triacylglycerols; heart contains choline plasmalogens, an alkyl lipid not present in other tissues. The close association with cancer cells of a specific class of lipids is of particular importance in the present investigation. Animal (Snyder and Wood, 1968) and human tumors (Snyder and Wood, 1969) contain elevated levels of ether-linked lipids (alkyldiacylglycerols). This ether-linked neutral lipid class is undetectable or is found only in trace quantities in normal mammalian tissues. The preponderance of ether lipids in neoplastic tissues raises some intriguing questions: can this biochemical abnormality be useful in determining the biologic potential of lesions of boarderline malignancy; what is their possible function in cell proliteration?

Books edited by Wood (1973) and Carroll (1975) contain indepth reviews of lipid metabolism in cancer cells, and a comparison of lipid metabolism

1079519

in normal and neoplastic growth has been compiled by Lee and Snyder (1977). Although the cellular functions of alkyl lipids have remained obscure since their discovery (Feulgen and Voit, 1924; Doreé, L909), the biosynthetic pathways of the ether-linked lipids are now well established (Snyder et al., 1969; Hajra, 1969; Hajra, 1970; Snyder, 1972; Kahn and Kolattukudy, 1973; Wykle and Snyder, 1976; Rock et al., 1978). An interesting distinction between cancer and normal tissues is that the anabolic pathways for the ether-linked lipids and their precursors are highly active in all cancer cells whereas the enzyme activities that cleave the ether bond are highest in normal tissues (Soodsma et al., 1970). A thorough investigation of these phenomenon utilizing the cell systems described will facilitate compariative studies of ether lipid metabolism in normal and neoplastic cells.

Lipids are important components of cellular membranes and membranes are important targets for phorbol esters (Wenner et al., 1974; Lee and Weinstein, 1978; Sivalc et al., 1972). Under conditions of in vitro exposure, TPA has been shown to effectively increase the specific activities of the plasma membrane enzymes Na^+-K^+-ATPase and 5'-nucleotidase; however, the microsomal enzymes NADH diaphorase and glucose-6-phosphatase were not affected (Sivak et al., 1972). In this respect, no information is available which defines the effects of in vitro TPA exposure on the lipid metabolic enzymes. The site of synthesis of alkyldiacylglycerols and ether-containing glycerophospholipids is the endoplasmic reticulum; if the plasma membrane is the primary site of TPA action, then alterations in the metabolism of ether-linked lipids may be a consequence of differentiation rather than a direct consequence of TPA exposure.

Ether lipid levels in cells can be manipulated by supplementing the growth medium with alkylglycerols such as hexadecylglycerol (Cabot and Snyder, 1980); this molecule serves as precursor mainly for synthesis of complex

1079520

ether-containing glycerophosphatides. On the other hand the amounts of alkyldiacylglycerols in cultured neoplastic cells has been shown by Scott et al., (1979) to be regulated at the level of glycolysis. Substitution of glucose for galactose produced very low levels of triacyl- and alkyldiacylglycerols; increasing the concentrations of glucose in the growth medium resulted in as much as a ten-fold increase in neutral glycerolipids. This system provides basic information on the regulation of ether lipids in relation to glycolysis, however few systems are available for studying ether lipid regulation as related to the degree of cell differentiation. In this regard the experimental systems and findings of the following investigators are of importance to the present investigation:

(1). In normal rat liver, Morris 7777 hepatoma and Morris 779A minimal deviation hepatoma (Snyder et al., 1969), the quantities of ether lipids were found to be proportional to growth rate of the tumor and inversely proportional to the degree of differentiation.

(2). The work of Howard et al., (1972) using a variety of transplantable hepatomas demonstrated a like correlation between ether lipids and tumor differentiation and found that the increased ether lipid levels were generally associated with glycerol phosphate dehydrogenase levels: the slowest growing, more differentiated tumors contained the highest amounts of enzyme. This enzyme would be important in regulating the levels of dihydroxyacetone phosphate, the glycerol source of alkyldihydroxyacetone phosphate. Additionally these investigators compared ether lipid levels in WI-38 fibroblasts and WI-38VA13A, a line derived from the diploid WI-38 by transformation with SV40 virus and found the transformed line to contain approximately 3-times the amount of ether-linked lipids.

(3). Brown et al., (1975) have shown that alkyldiacylglycerols occur in small quantities in the thymuses of both control and irradiated mice, however a 3- to 8-fold increase in this fraction occured in the thymic tumors which formed at 5 months postirradiation.

(4). Rossman et al., (1979) have isolated two clones from mouse preputial gland tumor. These clones were classified as (a) cells that undergo morphological differentiation in liquid medium to form round bodies filled with lipid vacuoles and (b) cells that do not differentiate. Lipid analysis of the former revealed higher amounts of triacylglycerols, alkyldiacylglycerols, sterol esters, and wax esters.

1079521

(5). The metabolism of ether-linked glycerolipids has been studied in primary rat tracheal epithelial cells and in a cell line derived from a benzo(a)pyrene-induced tumor (Scott et al., 1979). Growth of the primary epithelial cells in vitro resulted in the accumulation of alkyldiacylglycerols at levels comparable to those found in the tumor cell line; this class of lipids could not be defected in normal tracheal epithelium in vivo.

These studies clearly demonstrate that a correlation exists between alkyldiacylglycerol levels and cancer, and provide evidence which indicates that the metabolic anomalies are aligned with tissue and cell differentiation, however the role of lipids in neoplasia, their metabolic regulation during cell transformation and their use as possible markers during the early events of oncogenesis are points which merit further investigation. These studies would be facilitated by employing a system in which cellular differentiation can be carefully controlled and monitored.

1079522

C. Preliminary Studies: We have utilized LH-60 cells thus far in our studies to gain an overview of both polar and neutral lipid metabolism in hyperplastic (control) and TPA-differentiated systems. TPA caused an enhanced incorporation of ^{32}P into total cellular phospholipids. This stimulation was detected within 4 hours after treatment and preceeded the TPA-induced stimulation of lysozyme synthesis, phagocytosis, and morphological differentiation. Phospholipid fractionation revealed that the metabolic stimulation of ^{32}P incorporation was confied to the choline- and ethanolamine-containing phospholipids, whereas sphingomyelin, phosphatidylinositol and phosphatidylserine were unaffected. Treatment of the cells with 4-O-methyl-TPA, (1.7×10^{-10} M; 24 hours) an analog with poor tumor promoting activity, failed to elicit any alteration in phospholipid metabolism. Supplementation of the growth medium with different amounts of TPA for 24 hours resulted in a dose-related stimulation of ^{32}P incorporation into the cellular phospholipids. This dose-response correlates with the susceptibility of the cells to the induction of cell differentiation by TPA. Cells cultivated in the presence of TPA (1.7×10^{-10} M) for 24 hours showed a 3-fold increase over controls in the incorporation of [1,2-^{14}C]choline into phosphatidylcholine, results which were identical with the ^{32}P findings. However, when [2-^{3}H]-inositol was incubated with TPA-treated cells, a 2-fold increase in the phosphatidylinositol-associated radioactivity occured. This latter finding may be reflective of the modified pathway by which the anionic phosphoglycerides are synthesized. In addition to the effect of TPA on phospholipid metabolism in differentiating human leukemia cells we seek to investigate if other aspects of lipid metabolism occur during the differentiation process.

The data of Table I demonstrate the differential utilization of [^{14}C]acetate for the synthesis of cellular free fatty acids and their incorporation into neutral glycerolipids.

1079523

TABLE I

The utilization of ^{14}C-acetate for lipid synthesis in control and TPA-supplemented HL-60 leukemia cells

HL-60 cells were plated 1 day before treatment with TPA (1.7×10^{-10} M) and incubated simultaneously with 10 μCi [2-^{14}C]acetate, specific activity 54 μCi/μmol, for 24 h. The cells were harvested and total lipids extracted and analyzed as detailed in Methods. Data are based on dpm in lipid per mg cell protein; the lipid classes were resolved by thin-layer chromatography.

Incubation conditions	Label distribution			
	Phospholipids	Free Sterols[a]	Free Fatty Acids	Neutral lipids[b]
Control	351,111	19,875	27,796	42,425
TPA-supplemented	268,014	23,467	47,046	75,617

[a]Obtained from the unsaponifiable fraction in total lipid and verified as sterol by thin-layer chromatography.

[b]Neutral lipid refers to the combined triacylglycerol-alkyldiacylglycerol fraction.

In differentiation-induced cells versus control, a 70% increase occurred in the incorporation of acetate into the cellular free fatty acids. Likewise, the utilization of labeled fatty acids for neutral glycerolipid synthesis was markedly enhanced in the TPA-treated cultures (78% over control). Only a small increase of radioactivity in the sterol fraction occurred in treated cells. It is interesting to note the opposing effects of TPA-induced differentiation on phospholipid metabolism as characterized by ^{32}P and labeled polar head groups and the [^{14}C]acetate-labeled acyl groups. In the instance of the latter, a reduction of phospholipid radioactivity occurred in the TPA-supplemented cells.

1079524

As implied from the data of Table I, a stimulation of neutral lipid synthesis takes place during cellular differentiation. In order to determine if the isotope data reflect an actual increase in the net amounts of cellular lipid, total lipids from control and TPA-treated cells were fractionated and analyzed (Table II).

TABLE II

Lipid composition of control and TPA-treated HL-60 leukemia cells

Cells were incubated in the absence or presence of TPA (1.7×10^{-10} M) for 48 h. Cells were harvested, washed and lipids extracted. Amounts of neutral lipids were determined after resulution on thin-layer plates by photodensitometry.

Incubation conditions	Lipid composition[a]			
	Phospholipid	Sterols	Triacyl-glycerols	Alkyldiacyl-glycerols
Control	125	33	42	2.5
TPA-treated	134	36	69	3.9

[a]Values are given as μg lipid per mg protein.

On a per mg protein basis, the control cells contained 42 μg triacylglycerols versus 69 μg in the TPA-treated cells (64% increase). Although the ether-containing lipids account for a small amount of the total lipids in HL-60 cells, an increase similar to that observed in the triacylglycerols occurred in the TPA-exposed cells. The amount of the combined wax ester-cholesterol ester fraction in both control and treated cells remained identical. TPA treatment did not alter the amount of cellular sterols, nor was there a significant increase in the amount of phospholipid.

1079525

Our more recent studies have centered on ether lipid metabolism utilizing a labeled fatty alcohol precursor. An enhanced incorporation of hexadecanol into neutral lipids (123% over control) resulted in TPA-treated HL-60 cells. Since [1-^{14}C]hexadecanol can contribute to both the synthesis of ether- and ester-linked lipids, chemical reduction of the labile acyl groups showed ether lipid synthesis to be approximately 50% higher in TPA-exposed cells. These data reflect the labeled ether lipid content in total cellular lipids and do not differentiate ether-linked phosphoglycerides from neutral alkyl-diacylglycerols. Preliminary studies confined to alkyl group analyses of the phospholipid fraction (HL-60 cells) show that TPA has a stimulatory effect on plasmalogen production however alkyl group composition of control and treated cells remained similar.

Studies have now been initiated with cultured human melanoma cells which likewise can be induced to differentiation by treatment with TPA. A valuable feature of this cell model has been the establishment of a clone which does not display differentiation characteristics when grown in the presence of TPA. In the TPA-susceptible melanoma we have shown that treated cells contain greater amounts of neutral glycerides and unlike the human leukemia cells, display a significant increase in the cholesterol ester-wax ester component.

1079526

D. Methods of Procedure. The human promyelocytic HL-60 leukemia cells will be grown in bacterial plastic petri dishes (No. 1007, Falcon), in RPMI-1640 medium plus 20% fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Human HO melanoma cells and AO melanoma (TPA-resistent) will be cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cell numbers are determined from cell counts after excluding cells stained with trypan blue. Cells will be treated with TPA and other phorbol analogs 24 hr after subculture. Explicit details of cell culture procedure, TPA supplementation and evaluation of cell differentiation have been cited elsewhere (Huberman and Callaham, 1979; Huberman et al., 1979). TPA and its analogs will be added to culture media in DMSO so that its final concentration does not exceed 0.1%. This amount of DMSO has no effect on cell growth or differentiation. The oncogenic transformation of C3H/10T 1/2 cells has been described by Mondal et al., (1976). Briefly C3H/10T 1/2 cells are plated in 60 mm plastic dishes in 5 ml of basal medium (Eagle's) plus 10% heat-inactivated fetal calf serum and incubated in a humidified incubator at 37° in an atmosphere of 5% CO_2 in air. Twenty-four hr after plating, the medium is replaced with the same medium containing the initiator carcinogen (benzo(a)pyrene, 3-methylcholanthrene, 7,12-dimethylbenz[a]anthracene) and followed 4 days later by a nontransforming amount of TPA. By utilizing this procedure, the processes of initiation and promotion occur, and the involvement of lipids during oncogenic transformation can be studied in vitro.

1019521

Lipid isolation and analyses. Cellular lipids will be extracted by a modified method of Bligh and Dyer (1959) in which the methanol contains 2% glacial acetic acid. Both neutral and polar lipid classes can be resolved by thin-layer chromatography in a variety of proven solvent systems. The more refined separations include triacylglycerols and alkyldiacylglycerols, hexane/diethyl ether/acetic acid (80:20:1); cholesterol esters-wax esters-alkyldiacylglycerols, benzene (100%); and alkylglycerols-alk-1-enyl glycerols, diethyl ether/acetic acid/water (100/0.5/0.5). Quantitative analysis of the thin-layer resolved lipids is accomplished by H_2SO_4 charring of the chromatoplates at 200°C followed by photodensitometry according to Privett el al., (1965). When radiolabeled lipid precursors are utilized in cell culture systems, their distribution in the lipid extract will be determined by zonal profile scanning of the chromatogram (Snyder and Kimble, 1965). The alkyl group content of ether-containing phospholipids will be assessed by Vitride reduction to cleave labile esters followed by resolution of the products, alkylglycerols and alk-1-enylglycerols, on thin-layer plates.

Increasing the membrane-associated ether-containing phospholipids of cancer cells will be accomplished by chimyl alcohol supplementation to the growth medium as recently described (Cabot and Snyder, 1980). Evaluation of the effects of TPA on the chimyl alcohol treated cells will involve assessment of differentiation characteristics (HL-60 leukemia and HO-melanoma) in phorbol ester treated versus cells incubated in the absence of tumor promoter.

Experimental design. (a) TPA and a number of phorbol derivatives with varying promoter activities are readily available from commercial sources. In general the protocol for examining the lipid alteration in TPA-induced differentiating cells will involve culturing leukemia and melanoma cells with various concentrations of tumor promoter or suitable analog for varying time periods. Likewise the C3H/10T 1/2 cells will be oncogenically

1079528

transformed in culture as described by Mondal et al., (1976) and tested for type III transformed foci (Reznikoff et al., 1973). Leukemia, melanoma and mouse embryo C3H/10T 1/2 cells at various stages of treatment will then be characterized quantitatively and qualitatively for ether lipid composition. Transformed C3H/10T 1/2 cells consistently produce fibrosarcomas when injected into C3H mice. We will additionally take advantage of this procedure to investigate the ether lipid modifications in the animal-hosted tumor.

The evaluation of ether lipid metabolism in the cell systems described can be facilitated by utilizing radiolabeled fatty alcohol precursors. In particular [1-^{3}H]hexadecanol, synthesized by reduction of unlabeled palmitic acid with $LiAl^{3}H_{4}$ is advantageous over the [1-^{14}C]alcohol precursors in that biological oxidation of the former yields an unlabeled fatty acid. Therefore incorporation of the acid into acyl-linked lipids will not contribute to lipid radioactivity, and ^{3}H recovered in the cellular lipid extracts will be associated only with ether-linked phospholipids and neutral lipids (alkyl-diacylglycerols and unutilized fatty alcohol). Total cellular lipids can then be analyzed by thin-layer chromatography in the manner described. Close evaluation of the results gained from lipid compositional changes and cellular utilization of labeled fatty alcohols will provide necessary information for investigating lipid enzymological phenomenon occurring during cell transformation. These procedures will involve kinetic evaluation of the anabolic and catabolic enzymes of ether lipid metabolism and will be studied, in vitro, using cell homogenates and purified membrane fractions obtained by conventional differential centrifugation methods currently being used in our laboratory.

The enzymatic pathways that lead to the synthesis and degradation of ether-linked lipids have been thoroughly investigated by this laboratory and

1019529

others and the enzymes characterized. The in vitro enzyme assay procedures are routinely practiced and in many cases the substrates are available or can easily be synthesized via enzymatic or chemical means.

(b) Catabolic studies involving the cleavage of the alkyl moeity of ether-linked lipids will be carried out according to the optimum conditions described by Soodsma et al., (1972) and Snyder et al., (1973). The tetrahydropteridine-dependent O-alkyl cleavage enzyme can utilize glycero-lipid substrates that do not have a substituent at the sn-2 position (Snyder et al., 1973). We will use as substrate in these studies rac-hexadecylglycerol [1-^{14}C]ether which has been prepared commercially on special request.

(c) The first detectable product in the biosynthesis of the alkyl ether lipids, alkyldihydroxyacetone-P, is catalyzed by alkyl synthase in an alkyl-acyl displacement reaction that occurs between a long-chain fatty alcohol and akyldihydroxyacetone-P. This enzyme will be characterized in microsomes prepared from cells at various stages of treatment according to a recently described system (Brown and Snyder, 1979). Interpretation of the results obtained from cell culture in vivo studies of ether lipid synthesis can be substantiated by the in vitro kinetic evaluation of alkyl synthase; although the specific activity of alkyl synthase in tumors far exceeds that of normal tissues, we are presently unable to forecast the influence of chemically-mediated cell transformation on the activity of this enzyme.

Other key enzymes involved in the synthesis and regulation of the ether-linked lipids are the acyl-CoA reductases and fatty alcohol:NAD$^+$ oxidoreductases. The former enzyme holds an important role in ether lipid synthesis as the product fatty alcohols are substrates for the production of alkyl-DHAP and wax esters. The activity of acyl CoA-reductase can be surveyed in cell culture systems by adding [1-^{14}C]labeled fatty acids to

1019530

the growth medium and determining the amount of labeled fatty alcohol and alkyl-linkages in the cellular total lipid extract. In vitro assays, using microsomes as the enzyme source, will be performed according to the procedure described by Snyder and Malone (1970). The products formed in the assays will be identified by thin-layer chromatography using standards of fatty acids, fatty aldehydes, fatty alcohols, wax esters and alkylglycerols. Fatty alcohol:NAD^+ oxidoreductase is another important enzyme system involved in regulating the levels of long-chain fatty alcohols in tissues. Differences in the activity of fatty alcohol:NAD^+ oxidoreductase between control cells and cells at various stages of differentiation should likewise demonstrate the close association of this enzyme with ether lipid stasis and cell transformation. Fatty alcohol:NAD^+ oxidoreductase activity will be assayed according to the procedure described by Lee (1979).

The plasmalogen (alk-1-enyl) levels in the phospholipids are usually high in neoplastic cells (Veerkamp et al., 1961; Gray, G. M., 1963; Snyder and Wood, 1968; Snyder and Wood, 1969). Although the biological role of this monoenoic analog of alkylacylglycerophospholipids is not understood, the maintainence of the Δ1-double bond during cellular transformations should provide useful information regarding the possible function of plasmalogens. These lipids are integral parts of the cellular membrane matrix and their levels will be assessed simultaneously with the lipid compositional studies outlined in 3(a) of this section. Additionally, the in vitro activity of 1-alkyl-2-acyl-<u>sn</u>-glycero-3-phosphoethanolamine desaturase will be followed during cellular differentiation according to the method outlined by Wykle et al. (1972).

The first committed step in ether lipid biosynthesis is catalyzed by alkyl synthase and although this is a key regulatory enzyme, the synthesis of the complex macromolecular alkyldiacylglycerols and ether-linked phospholipids

1079531

is controlled by the branch-point enzymes acyl-CoA:1-alkyl-2-acyl-sn-glycerol acyltransferase and the choline- and ethanolamine-phosphotransferases. We have already demonstrated in our preliminary studies that TPA-induced differentiating leukemia cells contain significantly higher quantities of triacylglycerols and alkyldiacylglycerols. It is clear that alkyl synthase, acyl CoA reductase and fatty alcohol:NAD^+ oxidoreductase play important roles in providing the alkyl precursor for alkyldiacylglycerol synthesis, however the intermediate substrate alkylacyldiglyceride, can be utilized in vitro to compare and contrast its metabolic fate (neutral or polar lipids) during oncogenic transformation and TPA-induced cellular differentiation. The in vitro synthesis of alkyldiacylglycerols from 1-alkyl-2-acyl-sn-glycerols will be assayed using microsomal preparations according to the format of Blank et al., (1974). 1-Alkyl-2-acyl-sn-glycerol:CDP-choline (CDP-ethanolamine) choline (ethanolamine) phosphotransferase activity will be assayed using the cytidine diphosphate derivatives of choline or ethanolamine and 1-alkyl-2-acyl-sn-glycerol (Snyder et al., 1970).

In addition to the above cited reactions of ether lipid metabolism, we will also investigate the utilization of glucose in supplying the glycerol backbone of the alkyl lipids. This will be accomplished by supplementing [6-^{14}C]glucose to the growth medium of cells at various stages of differentiation. Total cellular lipids can then be extracted and analyzed for ^{14}C content.

The results of Scott et al. (1979) indicate that the levels of alkyldiacylglycerols in neoplastic cells can be regulated by the extent their precursors are formed from glucose. Elevated levels of DHAP may partially account for the accumulation of alkyldiacylglycerols in cancer cells, since it has been shown to be a precursor of the glycerol backbone of ether lipids (Hajra, 1969; Wykle and Snyder, 1969). We will explore the role that the

1079532

levels of endogenous DHAP play in contributing to the synthesis of the macromolecular ether-linked lipids in the cell systems described. Cells after various stages of treatment will be analyzed for DHAP levels in the manner detailed by Scott et al. (1979).

TPA has been shown to exert its effects at the membrane level; evidence for the direct stimulation of plasma membrane enzymes has been documented, whereas those of microsomal origin were not effected. The enzymes of complex ether lipid metabolism are microsomal, however it is important to test the effects of TPA directly on the in vitro systems employed for assay of alkyl synthase, acyl CoA reductase and fatty alcohol:NAD^+ oxidoreductase. Only minute amounts of TPA (1×10^{-10} M) are required to promote differentiation and alterations of lipid metabolism, and an in vitro modulation of lipid enzyme activities at this level would seem unlikely. These possibilities can never-the-less be investigated by including TPA, at several test concentrations, in the cell free incubation systems and measuring its effect on the key enzymes of ether lipid metabolism.

Very little information is available concerning the effects of tumor-promoting phorbol esters on lipid metabolism. The alignment of ether-linked lipids with neoplasia has been well documented, although the majority of these studies utilizing tumorous tissues were directed toward elucidating the biochemical pathways for their synthesis and degradation. The studies proposed in this application are intended to investigate the incidence of cellular differentiation, controlled in vitro, with the occurrence of a particular class of lipids closely associated with cancer, alkyl lipids. All of the areas outlined in this proposal can be tested experimentally in our laboratory as it is well equiped for studying all facits of lipid biochemistry. The

1079533

problem is realistic and the ideas straightforward. I believe use of the model systems outlined will much facilitate the mechanism by which ether lipids are so closely aligned with neoplastic growth.

We have a number of collaborative ties with scientists outside our group: cell biologists and toxicologists (Dr. Elie Huberman's group, Oak Ridge National Laboratories), organic chemists (Dr. Claude Piantadosi's group at the University of North Carolina, Chapel Hill), and mass spectroscopists at the Oak Ridge National Laboratory (Dr. William Rainey). These scientists could be helpful in solving some of the specialized problems that might arise.

The priorities for our proposed work will be essentially according to the order outlined in Section D. However preliminary data will be obtained through lipid compositional studies of cells after specific treatments, and these results will direct future project decisions so that major points can be explored clearly.

1079534

E. Facilities Available

Our laboratories are well equipped for the biochemical investigations outlined in this proposal. Some of the most significant equipment items include an infrared spectrophotometer, liquid scintillation spectrometers, a spark chamber for ^{14}C and ^{3}H detection on chromatograms, preparative ultracentrifuges with a B-XXIX zonal rotor and other conventional rotors, gas-liquid chromatographs, a thin-layer chromatography zonal scraper, an automatic freeze-dryer, a liquid nitrogen refrigerator, a specially designed tissue culture room with incubators, laminar-flow hoods, and a self-contained biohazard hood. We also have Beckman DU and Acta C-III spectrophotometers, photodensitometers, a complete line of thin-layer chromatographic equipment, disc gel electrophoresis apparatus, fraction collectors, columns for isolating and monitors for detecting proteins, and an analytical Model E. ultracentrifuge. In addition to the equipment in our laboratories, we have access to a nuclear magnetic spectrometer, a mass spectrometer, and an electron microscope in other sections of our Division and at the Oak Ridge National Laboratory. The mass spectrometer, which is connected to a gas-liquid chromatograph, is available to our group through a collaborative arrangement with Dr. W. T. Rainey, Jr., who is located at the Oak Ridge National Laboratory (Analytical Division). Our nuclear magnetic resonance studies are done in collaboration with Dr. B. Benjamin at the Oak Ridge National Laboratory (Chemistry Division). Electron microscopic and pathologic services are readily available in our Division and at the Oak Ridge National Laboratory. Collaboration in all of these areas has been amply demonstrated in our published work.

"USE OF DOE FACILITIES AND DOE CONTRACT REQUIREMENTS"

This research grant application includes a segment of activity which would be performed in facilities of the U. S. Department of Energy and governed by an existing contract between Oak Ridge Associated Universities (ORAU) and the DOE. The DOE has reviewed this proposal and has concurred in ORAU conducting the described work in the DOE facilities made available for biomedical research, subject to payment to the DOE by ORAU from NIH funds of the applicable direct and indirect cost of the work (not including any charge for the use of DOE facilities) as determined by the provisions of the DOE's contract with ORAU. It is believed that in large measure the requirements of the DOE contract parallel conditions which NIH ordinarily applies to its grants. In the event of differences between NIH grant terms and the DOE contract terms, ORAU is agreeable to meeting both to the extent that they are not in conflict, and to applying those most favorable to the United States Government where this is involved. If NIH is aware of problems which such an approach would produce or suggest, ORAU upon receipt of such advice would refer the matter to the DOE for direct resolution with NIH.

By way of general information, ORAU's contract with the DOE is a cost-type contract financed under a Government-fund account. The specific contract work is formulated in cooperation with the DOE and authorized within general guidelines in the contract. Contract terms include DOE responsibility for Government ownership and control of inventions, data, and other research products. Ownership of all equipment and facilities acquired by ORAU with DOE funds is vested in the U. S. Government at the time of acquisition. The contract also contains all the terms generally common to Government contracts of the type under which ORAU conducts research operations in Government-owned facilities.

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079535

NAME (last, first, middle initial)
Cabot, Myles C.

SOCIAL SECURITY NUMBER
[redacted]

Continuation page

F. Collaborative Arrangements: None

G. Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

Principal Investigator

Date

DO NOT TYPE IN THIS SPACE-BINDING MARGIN

1079536