948
(R)

712372

Oak Ridge
Associated
Universities

Post Office
Oak Ridge
Telephone

October 27, 1981

Dr. William R. Bibb, Director
Research Division
Department of Energy
Oak Ridge, TN 37830

Subject: GRANT APPLICATION TO NIH FOR A PROJECT ENTITLED *LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA*

Dear Dr. Bibb:

Enclosed are three copies of the subject grant application to NIH. The proposed project will be carried out under the direction of Dr. Myles Cabot. Draft copies of this application were forwarded to your office for review on September 18, 1981, and approval for formal submission was given by Dr. Richard Benson on September 29.

Should this project be approved by NIH, the work will be carried out under policies and procedures previously established between ORAU and DOE.

We will keep you advised of NIH action on this proposal.

Sincerely,

William E. Felling

William E. Felling
Acting Executive Director

RYAN:br

Enclosures None rec'd - no files

REPOSITORY Oak Ridge Operations Records Holding area
COLLECTION Documents 1944-94
BOX No. B-87-9 Bldg. 2714-H
FOLDER 20-89-81 NIH

20-89-81

1079605

U 5/36

Oak Ridge
Associated
Universities

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

Executive
Office

October 27, 1981

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. This grant is being submitted in place of an earlier submission by the same title and assigned NIH No. 1 R01 CA 29153-01. We request that the earlier application be withdrawn from consideration. The enclosed proposal has been re-written based on critical review of the earlier version. Bob Hawkins advised that Dr. Cabot request this substitution.

Oak Ridge Associated Universities is a nonprofit corporation sponsored by 51 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 on pages 10 and 11 of this 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
[redacted] E. FELLING

William E. Felling
Acting Executive Director

br

Enclosures

cc: Dr. Thaddeus Domanski
Chemical and Physical Carcinogenesis Branch
Division of Cancer Cause and Prevention
National Cancer Institute
Bethesda, Maryland 20205

bcc: Dr. William R. Bibb DOE ORO (3)
Executive Office
W. F. Countiss
M. Cabot
B. Ryan

1079606

FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT [x] NO [ ] YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
CABOT, Myles C.

3b. SOCIAL SECURITY NUMBER
[redacted]

3c. MAILING ADDRESS (Street, city, state, zip code)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

3d. POSITION TITLE
Biochemist

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biological Chemistry

3f. TELEPHONE (Area code, number and extension)
615, 576-3122

3g. MAJOR SUBDIVISION
Medical and Health Sciences Division

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
[x] NO [ ] YES (If "YES," form HEW 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
[x] NO [ ] YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: 1 July 1982 Through: 30 June 1985

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$220,000

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$69,400

9. PERFORMANCE SITES (Organizations and addresses)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
[x] NO [ ] YES - Previously reported
[ ] YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

Third Congressional District

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)
Code 6 0 Description:
(Medical and Health Sciences Division)

13. ENTITY IDENTIFICATION NUMBER
1620476816A1

14. TYPE OF ORGANIZATION (See instructions)
[x] Private Nonprofit
[ ] Public (Specify Federal, State, Local):

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615, 576-3056)

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
William E. Felling
Acting Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615, 576-3300)

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)
Myles C. Cabot

DATE
Oct 22, 1981

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)
William E. Felling

DATE
OCT 2 1981

PHS-398
Rev. 10/79

1079607

DEPARTMEN _F HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

ABSTRACT OF RESEARCH PLAN

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION (Same as Item 11, page 1)
Oak Ridge Associated Universities, P. O. Box 117
Oak Ridge, Tennessee 37830

TITLE OF APPLICATION (Same as Item 1, page 1)
LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

Myles C. Cabot, Ph.D., Biochemist, Biological Chemistry, Medical and Health Sciences Divisio

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

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 of 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, CTP-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. As an essential corrolary to the proposed experiments, we will conduct lipid metabolic studies employing a human leukemic cell line that does not express morphological or biochemical macrophage-like markers when exposed to TPA. The ultimate objective is to understand what factors govern the TPA-induced stimulation of phosphatidylcholine metabolism in differentiating cells, and determine if a link exists between phospholipid stimulation and tumor promoter-induced differentiation.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

None

1079608

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

SECTION 1. **PAGE NUMBERS**

Face Page, Abstract, Table of Contents	1-3
Detailed Budget for First 12 Month Budget Period	4
Budget Estimates for All Years of Support	5
Biographical Sketch-Principal Investigator/Program Director *(Not to exceed two pages)*	7
Other Biographical Sketches *(Not to exceed two pages for each)*	
Other Support	9
Resources and Environment	10

SECTION 2.

Introduction *(Excess pages; revised and supplemental applications)*	
Research Plan	
A. Specific Aims *(Not to exceed one page)*	12
B. Significance *(Not to exceed three pages)*	13
C. Progress Report/Preliminary Studies *(Not to exceed eight pages)*	16
D. Methods	18
E. Human Subjects, Derived Materials or Data	24
F. Laboratory Animals	24
G. Consultants	24
H. Consortium Arrangements or Formalized Collaborative Agreements	24
I. Literature Cited	24
Checklist	28

SECTION 3. Appendix *(Six sets) (No page numbering necessary for Appendix)*

Number of publications: 1 Number of manuscripts: 1

Other items (list):

Two curriculum vita
One table

Application Receipt Record, form PHS 3830
Form HEW 596 if Item 4, page 1, is checked "YES"

1079609

PRINCIPAL INVESTIGATOR/PROGRAM DIRE ?: CABOT, Myles C.

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD
DIRECT COSTS ONLY

FROM	THROUGH
07/01/82	06/30/83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)

NAME	TITLE OF POSITION	TIME/EFFORT %	TIME/EFFORT Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
Cabot, Myles C., Ph.D.	Principal Investigator	50	20	15,000	3,000	18,000
Welsh, Clement J.	Research Associate	50	20	9,430	2,120	11,550
TO BE NAMED	Postdoctorate or Research Technician	100	40	18,000	3,600	21,600
	SUBTOTALS →			42,430	8,720	51,150

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

Coulter Counter	$4,650	4,650

SUPPLIES (Itemize by category)

Tissue Culture Ware	$2,300	
Cell Culture Media & Serum	4,000	
Chemicals	1,700	
Radiolabeled Compounds	4,600	
		12,600

TRAVEL	DOMESTIC	1,000
	FOREIGN	
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

TOTAL DIRECT COSTS (Also enter on page 1, item 8) → $ 69,400

1079610

PRINCIPAL INVESTIGATOR/PROGRAM DIR')R: CABOT, Myles C.

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		51,150	56,300	61,900		
CONSULTANT COSTS						
EQUIPMENT		4,650	0	0		
SUPPLIES		12,600	13,900	15,500		
TRAVEL	DOMESTIC	1,000	1,000	1,000		
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES		0	500	500		
TOTAL DIRECT COSTS		$69,400	$71,700	$78,900		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 7) → $ 220,000

JUSTIFICATION (Use continuation pages if necessary): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

Recurring Increseas in Personnel Costs computed at 10%.

Equipment Justification:

1st Year - Coulter counter model ZF Serial 6600338 - Our present Coulter counter (model B) is an old, tube model with an excessive amount of down-time. Accurate, reproducible and rapid cell number assessment is an essential guideline for the current project, as ell number data will be used for differentiation criteria. The model ZF is the most economical counter of the series and offers all the features we require.

Personnel Justification:

Clement Welsh (Research Associate). Mr. Welsh was trained by me and works closely with me on the HL-60 project. He will be carrying out lipid composition analyses (These are time consuming and require skill). In addition, those duties which Mr. Welsh will aid include cell culturing, induction of differentiation protocol in vitro enzyme assay and laboratory management.

1079611

Postdoctorate or technician (to be named). This person will devote 100% of their time to project research; the position will require a working background in cell biology. The major duties of this position will be assessment of cell differentiation criteria (both morphological and biochemical) using the induction agents listed at various doses over various time intervals. In addition, in vitro enzyme assays of the phosphatidylcholine-metabolizing activities will be an important facet of this position.

1079612

PRINCIPAL INVESTIGATOR PROGRAM DIRE R: CABOT, Myles C.

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Myles C. Cabot	Biochemist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

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

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

1976-1978: Damon Runyon-Walter Winchell Postdoc. Fellow in Cancer Research, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

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

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.

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, 838-846, 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 617, 410-418, 1980.

Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. *Alterations in lipid metabolism induced by 12-O-tetradecanoyl-phorbol-13-acetate in differentiating human myeloid leukemia cells.* Cancer Res. 40, 3674-3679, 1980.
Cancer Res. 40, 3674-3679, 1980.

Cabot, M. C. and Goucher, R.R. *Chaulmoogric acid: Assimilation into the complex lipids of mycobacteria.* Lipids 16, 146-148. 1981.

Cabot, M. C. and Lumb, R. H. *The activity of a low temperature lipase in the larvae of Sarcophaga bullata (Diptera: Sarcophagidae).* Comp. Biochem. Physiol. 68B, 325-328, 1981.

Cabot, M. D. and Welsh, C. J. *Ether lipid studies in mouse C3H/10T1/2 cells and a 3-methylcholanthrene transformed clone.* Arch. Biochem. Biophys. 211, 240-244, 1981

Cabot, M. C. and Welsh, C. J. *Fatty acid metabolism in phorbol ester-differentiating human leukemia cells.* Cancer Res. (in press)

Cabot, M. C., Welsh, C. J., and Snyder F. *Modifying the levels of ether-linked lipids in L-M cells alters growth and choline utilization.* Exptl. Cell Res. (Submitted).

1079614

OTHER SUPPORT

(USE CONTINUATION PAGES IF NECESSARY)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications pending review and/or funding; (3) applications planned or being prepared for submission. Include all Federal, non-Federal, and institutional grant and contract support. If none, state "NONE." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent grant and the subproject and give the annual direct costs for each.) Briefly describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, justify and delineate the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) ACTIVE SUPPORT:

Investigator currently has no individual research support. He is supported under other larger projects:

A. American Cancer Society, Grant No. BC-70L; "Metabolism, Regulation and Function of Ether-Linked Glycerolipids and Their Precursors in Cancer Cells"; Dr. Fred Snyder: Senior Investigator; 20% Effort: $58,150 annual direct costs and entire period support; Purpose is to study the metabolism and regulation of ether- and ester-type glycerolipids in selected cancer cells.

B. NIH; Grant No. 5 R01 CA 11949-12; "Ether Lipids in Cancer - Enzyme Mechanisms"; Dr. Fred Snyder: Senior Investigator; 25% effort; $57,404 annual direct costs; $305,031 TDC entire period of support; Purpose is to purify key enzymes involved in the metabolism of ether linked lipids, so their kinetic properties and reaction mechanisms can be established.

C. DOE; Contract No. DE-AC05-76OR00033; "Lung Surfactant, Membrane, Lipids and Energy Biohazards"; Dr. Fred Snyder: Senior Investigator; 55% effort; $223,549 annual direct costs and entire support period; Purpose of research focuses on health problems created by newer energy technologies; study the effect of chemical toxicants, carcinogens and radiation on systems being investigated.

(2) NONE.

(3) NIH; Grant Application; "Membrane lipids and differentiators of leukemia"; Dr. Myles C. Cabot, Senior Investigator; 50% effort; $71,000 1st year annual direct costs: $413,900 TDC total support period; the purpose is to investigate inducers of leukemic cell differentiation and determine if there exist lipid metabolic alterations that are common to the differentiation process. Secondly, do lipids play a role in cellular differentiation or are the induction agents causitive?

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: CABOT, Myles C.

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe facilities at other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include a description of the nature of any collaboration with other organizations and provide further information in the RESEARCH PLAN.

[X] Laboratory: My laboratory is approximately 17 x 25 ft.; it is qell equipped for carring out experiments and has two offices adjacent, one of which contains a freezer and refrigerator. The lab is located in the biochemistry department of the Medical and Health Sciences Division with analytical and preparative capacities at hand.

[ ] Clinical:

[ ] Animal:

[X] Computer: We have immediate access to computer ware.

[X] Office: My office is located adjacent to my laboratory.

[X] Other (Tissue culture): We have two culture facilities, on e located directly across the hall from my laboratory. The human cell culture lab is located upstains (approx. 45 seconds away).

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location, and pertinent capabilities of each. Lipid nitrogen refrigerator; thin-layer chromatographic equipment, including zonal scraper and spark chamber; photodensitometer; preparative and analytical centrifuges; Model E ultracentrifuge, liquid scintillation spectrometer, gas-liquid chromatographs; HPLC; Beckman DU and Acta C-III spectrometer; Cahn electrobalance; protein isolation equipment. All the equipment is in excellent working order and located in the labs adjacent to my laboratory. Service contracts are active.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

We have on board secretarial staff, machine and electronics shop, access to electron microscopy, mass spectrometry; consultants in the areas of cell biology, cytogenetics, organic chemistry. The support services are readily available.

'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.

1079616

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.

1079617

[redacted]

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 (1-3). However, it is significant to note that phorbol esters, when used in the absence of initiators induce rather than inhibit differentiation in human leukemia (HL-60) cells (4) and stimulate differentiated functions in human melanoma cells (5). We have recently begun studies of the alterations in lipid metabolism elicited by treatment of HL-60 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 counterparts, 1-O-alkyl-2-acylglycerophosphatides, occur at characteristically high levels in cancer cells (6,7).

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-enylglycerophosphatides. 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 conducted. These studies will be carried out 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 (polar head group, hydrocarbon moieties) 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. We endeavor to determine if phosphatidylcholine turnover is linked to the induction of differentiation. This query can in part be answered by comparing ^{32}P and [^{14}C]choline utilization in 1) cells induced to differentiate by treatment with other active induction agents, dimethyl sulfoxide and retinoic acid, and 2) in leukemia cells that do not undergo differentiation when exposed to TPA. Finally, we wish to define the location of the induction by TPA leading to the lipid alterations by examining the assimilation of labeled precursors in isolated subcellular fractions.

1079618

B. SIGNIFICANCE

Berenblum (8) discovered that a single application of benzo(a)pyrene 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 (9). TPA, the most abundant and most active tumor-promoting agent of croton oil, has been used widely in the two-stage mouse skin carcinogenesis system (10) 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 (22). Recent studies have shown that when tumor promoters are used singly they inhibit spontaneous and induced differentiation in murine erythroleukemia cells (12-14), mouse neuroblastoma cells (15), 3T3 fibroblasts (16), and avian myoblasts (17). Conversely, studies with HL-60 cells (4, 18) and human melanoma cells (5) 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 (19) and Balmain and Hecker (20) examined the relationship between TPA-induced tumor formation in mouse epidermis and phospholipid metabolism and found that TPA stimulated the early synthesis of phosphatidylcholine and phosphatidylethanolamine. In bovine lymphocytes TPA causes an enhanced labeling with [Me-^{3}H]choline of phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin (21). 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 (22). 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-associated alterations in a variety of systems: these include modifications of lipid microviscosity in lymphoblastoid cells (23), inhibition of adipose conversion in 3T3 fibroblasts (16), stimulation of secretion of disaturated phosphatidylcholine from alveolar type II cells (24), enhancement of phospholipase activity and prostaglandin production (25, 26), and alterations in ganglioside metabolism in human melanoma cells (5). We have recently shown (27) that TPA-induced differentiation of HL-60 cells is accompanied by a stimulation of phospholipid metabolism (before the markers of differentiation are expressed), enhanced incorporation of acetate into free fatty acids and neutral lipids, an increase in the amount of cellular triacylglycerols, and a selective incorporation fatty alcohol into triacylglycerols and their ether-containing analog, alkyldiacylglycerol.

Whereas, a number of investigators have shown that TPA supplementation to cell cultures causes myriad alterations in lipid metabolism, the effects of phorbol esters on cellular differentiation provide a morphological marker for correlating with the TPA-induced modifications of lipid metabolism. In this

1079619

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. HL-60 cells are extremely useful for such studies, since a large population of the cells (more than 90%) can be induced to differentiate by phorbol esters (4). TPA-induced differentiation in these cells has been characterized by morphological changes, decreased cell division, an increase in the percentage of phagocytizing cells, the attachment of the cells to the substratum (4), and stimulation of synthesis and extracellular release of lysozyme. This last finding is of interest since TPA can cause lysozyme release from peripheral blood polymorphonuclear leukocytes, but not from alveolar macrophages (28, 29).

Although the action of TPA as an inducer of cell differentiation has been the object of considerable study, a variety of compounds, unrelated chemically, have been shown to induce morphological and biochemical alterations in numerous cell lines. DMSO induces erythroid differentiation in mouse erythroleukemia (Friend) cells and the morphological, biochemical, and immunological changes induced have been well characterized (30-32). The differentiation-inducing effect of DMSO has recently been demonstrated in human cells (5, 33-35), and retinoic acid, another potent differentiator, has been shown to induce maximal differentiation (approximately 90%) of HL-60 cells at a concentration of 1 M, an amount much less than the concentration needed for DMSO to produce similar differentiation (36). These studies suggest that HL-60 cells share common target sites for the induction of differentiation by these compounds.

Lipids are important components of cellular membranes and membranes are important targets for phorbol esters (37-39). 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 (39). However, no 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. Additionally, a shortcoming of previous studies has been the failure to define whether the stimulation of phosphatidylcholine metabolism is linked to the induction of differentiation in HL-60 cells. Although TPA has been shown to significantly alter phosphatidylcholine metabolism in HL-60 cells and other cell lines, DMSO and retinoic acid, effective agents of induction, have not been investigated. Kinzel et al. (22) have shown that DMSO enhances the incorporation of [^{3}H]choline into HeLa cells. DMSO was not as effective as TPA; however, these investigators used 0.5%, an amount which is not sufficient to induce maximal differentiation of HL-60 cells (33).

Phosphatidylcholine, a structural component of animal cells, has been implicated in the modulation of certain enzyme activities (40). Phospholipid metabolism has also been linked to events leading to the stimulation of growth (41-45). 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

1079620

the lack of an appropriate model system wherein modifications of phospholipid metabolism can be correlated with cellular differentiation by 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. Our investigations, as well as studies by others, demonstrate that one of the earliest events elicited by TPA is the stimulation of phosphatidylcholine metabolism. This information reinforces the theory that membranes are an important target of phorbol esters, and thus could modulate signals that initiate cellular responses.

The major pathway for phosphatidylcholine biosynthesis was elucidated by Kennedy (46) and his co-workers. 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 (47). Lands and his colleagues were the first to demonstrate the deacylation-reacylation cycle of phosphatidylcholine synthesis (48, 49). Although of minor significance, phosphatidylcholine can additionally be synthesized by a base exchange reaction (50). Of the enzymes involved in phosphatidylcholine biosynthesis, it now appears that the regulated step is catalyzed by the enzyme, CTP-phosphocholine cytidylyltransferase (51-52). 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 phosphocholinetransferase and converts diacylglycerol and CDP-choline into phosphatidylcholine. With the evidence that tumor promoters elicit remarkable alterations in the metabolism of phosphatidylcholine in bovine lymphocytes (21) and HeLa cells (22), there has been little advancement regarding the mechanism responsible for this effect. It has been shown that one of the earliest responses elicited by tumor-promoting phorbol esters is the alteration of phospholipid metabolism (11, 27, 54-57). 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 (41-45).

The following points need to be considered in proposing the mechanism by which TPA stimulates phospholipid metabolism. Wertz and Mueller (21) have shown that 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 the phospholipid metabolizing enzymes during TPA-induced cellular differentiation will also lend information regarding the role of phosphatidylcholine in cell growth and differentiation.

1079621

[redacted]

C. PRELIMINARY STUDIES

Our initial results that document dramatic alterations in lipid metabolism occurring in TPA-differentiated HL-60 cells have been published (27) (reprint, Appendix I). Briefly, this work shows that TPA-induced differentiation of HL-60 cells is accompanied by a stimulation of phospholipid metabolism before the markers of differentiation are expressed. Phospholipid metabolism was assessed by differences in the utilization of labeled precursors, ^{32}P and [^{14}C]choline, in control (untreated) and TPA-treated cells. Although other changes in lipid metabolism were demonstrated, we believe, from our most recent studies (manuscript, Appendix I) that the increased levels of triacylglycerols occurring in treated cells is a characteristic inherent to the differentiated cell, whereas the flux in phosphatidylcholine metabolism is early in the event of differentiation. This may suggest that the stimulation of phosphatidylcholine metabolism by TPA is linked to the induction of differentiation.

The effects of TPA-induced differentiation on phospholipid metabolism have been limited to studies using labeled precursors of the polar head groups, thus little is known about the effects of phorbol ester-induction on fatty acid metabolism. A topic of ongoing investigation has therefore been in the direction of acyl group metabolism of the phospholipids (manuscript, Appendix I). As the data and description of these experiments are outlined in detail, only a brief description of that investigation follows. In cells incubated with labeled fatty acids for 1 hr, the percent distribution of lipid radioactivity is highest in phospholipids of undifferentiated cells, whereas in TPA-supplemented cultures, substantially more label is associated with triacylglycerols. This trend was confirmed by data derived from analysis of lipids in undifferentiated and TPA-treated cells (8×10^{-10} M TPA, 48 hr), where in the latter, the amount of triacylglycerols increased 3.2-fold. The increase in the amount of cellular phospholipids was not as pronounced. Whereas a marked stimulation of the incorporation of ^{32}P and choline into phosphatidylcholine has been shown to occur in TPA-differentiated cells, acyl group metabolism in phospholipids is affected to a much lesser degree. Thus the turnover of the polar portion of the molecule appears to be stimulated more than the hydrophobic moieties of phospholipids. An additional interesting observation on fatty acid metabolism in differentiated cells was the finding that the desaturation of stearic acid (conversion of [1-^{14}C]stearic acid to [1-^{14}C]oleic acid by cell cultures) was greatly reduced compared to control activity. A corrolary reflecting the cellular consequences of this phenomenon was shown by marked differences in acyl group composition of triacylglycerols in differentiated cells, which contained decreased amounts of unsaturates compared to the triacylglycerol fatty acid profile of undifferentiated cells.

We have endeavored to determined if the rate of uptake of free fatty acids is higher in differentiated cells. At early times after TPA exposure (1 to 4 hr), there was no stimulation of the cellular uptake of labeled fatty acid; however, differentiated cells (24 to 48 hr TPA-treated), when exposed to labeled acids, contained more radioactivity than undifferentiated cells. This phenomenon was, however, variable and dependent on the fatty acid supplemented, with arachidonic acid showing the greatest uptake differential. When [1-^{14}C]-palmitic acid and TPA were added simultaneously to cultures and incubated for 48 hr, the cells contained approximately twice the radioactivity (dpm/mg cell

1079622

protein) compared to cells labeled in the absence of TPA for the same time period. Qualitative analyses of the lipids in the control and TPA-treated cells showed the latter to contain a 4-fold increase in the amount of triacylglycerol-associated radioactivity.

The study of acyl group metabolism in phospholipids of differentiated cells has only been touched on; we have confirmed that the phosphatidylcholine fraction isolated from TPA-differentiated cells, labeled with [1-^{14}C]stearic acid, contains more radioactivity than ethanolamine-containing phosphoglycerides (approximately 2 times). Our preliminary data on fatty acid metabolism coupled with our previous findings on polar head group utilization has yielded significant information to form a natural backdrop for continued studies. We are most excited with our results of late: by utilizing the culture system described by Brietman et al. (58), we now have HL-60 cells (passage 45) growing in the absence of serum. The serum required to grow cells is a rich source of fatty acids. It has always been an advantage for one interested in lipid studies to have a versatile system wherein cells can be grown either in the presence or absence of serum. Furthermore, we have just demonstrated that these cells, grown in the absence of serum, can be induced to differentiate into a highly enriched population of macrophage-like cells (this is the first report of this phenomenon, manuscript in preparation). This is a significant finding, in view of the fact that serum-grown cells treated with TPA have been morphologically characterized as both macrophage-like and granulocytic, even though they possess high acid phosphatase levels (59), an enzymatic marker for macrophages. The acyl group composition of phospholipids and triacylglycerols from cells grown with serum and in a serum-free medium is shown in Table I (Appendix II). Compared to the fatty acid composition of serum-grown cells, the serum-free cells demonstrate a relatively simple acyl profile; most notably is the near absence of polyunsaturates with 16:0 + 16:1 + 18:1 comprising about 80% of the total species, and as shown by the DMA derivatives, plasmalogen levels are 5-fold lower. In view of the fact that HL-60 cells can be passaged in the absence of serum and differentiate in the presence of TPA, this system will be extremely useful in studies aimed at acyl group metabolism of phosphatidylcholine and ether lipid modulations in differentiation.

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 the fields of lipid metabolism, membrane biochemistry (membrane-enzyme-substrate interactions, membrane modification), and tissue culture. As a result of my training and current laboratory environment, I feel well qualified to pursue the studies on the effects of TPA on ether lipid metabolism. 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.

1079623

D. METHODS

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

The modification of phosphatidylcholine metabolism is the first lipid-related event 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 cellular lipid composition of control and TPA-exposed cells, varying the concentration and time of exposure to TPA, to assess the effects of induced differentiation on the levels of phosphatidylcholine, phosphatidylethanolamine, inositol- and serine-containing phosphoglycerides, triacylglycerols, alkyldiacylglycerols, and cholesterol. These experiments will in turn be carried out with cells grown in serum-free media. 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. Ether phosphoglyceride levels will also be extensively analyzed in isolated plasma membrane and nuclear fractions. The general protocol for lipid compositional analyses during induced differentiation will involve growing the cells in media containing TPA (8×10^{-10} to 1.6×10^{-8} M) for periods up to 3 days. The extracted lipids (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 incorporation of ^{32}P and [^{14}C]choline into phosphatidylcholine. We will extend these studies to investigate the effects of TPA on a) the coulping of choline transport and phosphorylation, b) phosphatidylcholine acyl group metabolism, and c) de novo synthesis of phosphatidylcholine from labeled glycerol and glycerol precursors. 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.

Radiolabeled palmitic, stearic, oleic, and arachidonic acid will either be added to the growth medium (plus serum, serum-free) 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 48 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 phosphate-buffered saline, isolated by brief centrifugation, and the lipids extracted and analyzed as detailed. Purified plasma membrane, microsomal, and nuclear fractions will additionally be examined.

1079624

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 concentration of TPA in the incubation medium to more thoroughly assess the effects of TPA on transport and metabolic fate of the labeled precursors. Because it is important to define whether the stimulated phosphatidylcholine turnover observed during TPA induction is linked to differentiation, we will carefully examine ^{32}P and [^{14}C]choline uptake and utilization in cells induced with dimethylsulfoxide and retinoic acid. Transport of labeled choline will be measured by the disappearance of radioactivity from the cell media. 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 the cells will be washed in fresh medium and replated. At various times following the pulse labeling, the cells will be harvested and the water-soluble and organic-labeled compounds will be 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. (53).

As little is known on how TPA affects the acyl group metabolism of phospholipids, investigation 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 fatty acids into cellular phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. Acyl group turnover will be assessed by labeling a series of control and TPA-treated cultures for 1 hr. The cultures will then be replated in fresh media and selected at various times to determine the radiospecific activity in the choline-, ethanolamine-, inositol-, and serine-containing phospholipids.

De novo synthesis of phosphatidylcholine from labeled precursors (glycerol, glucose) will be evaluated in control and TPA-differentiating cells to determine if TPA exposure alters the utilization of these molecules for the synthesis of phosphatidylcholine. Double-labeled 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 (60, 61); 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 phospha-

1079625

tidylcholine fraction to remove labile esters followed by isolation of labeled glycerol (62). 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 untreated HL-60 cells. Once these conditions are established, the activities of the enzymes will be compared in control and TPA-exposed cells.

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 (63).

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 (64) as modified by Weinhold et al. (65).

c. Phosphocholinetransferase. The phosphocholinetransferase actiivity 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. (66) and modified by Vance and Burke (67) 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 (67); 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 (68) 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 (69).

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 (53) in control and TPA-exposed cells.

1079626

5. TPA effect on phospholipid metabolism in myeloid blast cells

An important corrolary to the proposed experiments will be established by using human leukemia cells that are resistant to TPA-induced differentiation. Cells of the line K562 do not express macrophage-like characteristics (morphology, adhesion, spreading, phagocytosis, NBT reduction, lysozyme secretion) when exposed to TPA (70). We will use these cells in a series of experiments, centering on ^{32}P and [^{14}C]choline utilization, following protocol as detailed in D. Part 2 (Studies of phosphatidylcholine metabolism using radiolabeled precursors). These experiments will provide information that will clarify the extent to which TPA can modify phospholipid metabolism per se versus alterations in lipid metabolism that accompany the differentiation process.

6. Specifics of procedure

a) Cell culture, differentiation, and subcellular fractionation

The HL-60 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). K562 human leukemia cells are presently cultured in our laboratory; Dr. B. Lozzio (University of Tennessee Memorial Research Center, Knoxville) has agreed to donate his K562 line for future experiments. 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. The concentration of TPA in the culture medium will be 8×10^{-10} M. This concentration, in our hands, has been sufficient to induce differentiation; however, experiments will be conducted using a range of concentrations, 10^{-9} to 1.6×10^{-8}, the highest being the amount employed by most investigators. Explicit details of cell culture procedure, TPA supplementation, and evaluation of cell differentiation have been cited elsewhere (4, 27). TPA will be added to culture media in DMSO so that its final concentration does not exceed 0.01%. This amount of DMSO has no effect on cell growth or differentiation. Terminal differentiation induced by other agents (33) will be carried out by incubating cells for 6 days in 1.25% DMSO, in a like manner for cells grown in serum-free media (58), or in media containing retinoic acid (1×10^{-6} M) as described by Breitman et al. (36). We have shown in our previous study that the stimulation of phosphatidylcholine metabolism preceeds the induction of differentiation (27). 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, inducer concentration, and degree 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. Cellular differentiation (morphological) will be assessed periodically by performing differential counts on Wright-stained cell preparations and based on the percentage of myelocytes, metamyelocytes, and banded and segmented neutrophils and macrophages. The control population consists primarily of promyelocytes (90%). Other criteria include cell attachment and cessation of growth. Macrophage populations will

1079627

be assessed. Biochemical markers for differentiation include NBT dye reduction (35. 71), phagocytosis of Candida albicans (33), lysozyme release (29), and specifically in the case of TPA, acid phosphatase activity for the macrophage-like differentiation produced (59).

Plasma membranes will be isolated following a published protocol specific for HL-60 cells (72). Nuclei will be isolated according to a procedure described for guinea pig polymorphonuclear leukocytes (73). This procedure employs the homogenization of cells in a medium that is slightly hypotonic followed by immediate restoration of isotonicity. By this method, the integrity of subcellular organelles is maintained. The purity of the subcellular fraction will be established by assay of classical marker enzymes. We will additionally utilize the methods of De Pierre and Karnovsky (73) and Michell et al. (74), which provide invaluable techniques for the isolation and characterization of subcellular fractions.

b) Lipid isolation and analyses

Lipids will be extracted from control and TPA-exposed cells by a modified method of Bligh and Dyer (75) 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 (76, 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 commercial standards will be used for the identification of individual lipid classes. The more refined separations include triacylglycerols and alkyldiayl-glycerols, 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 for 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. (77). 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 (78). 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 (79). For phospho-lipid 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 (80).

Changes in the acyl group composition of phosphatidylcholine may indicate altered physical properties of the membranes in control versus TPA-exposed cells. To investigate the effects of TPA on acyl group composition, the phos-phatidylcholine 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

1079628

at 100°C (81) and analyzed by gas-liquid chromatography according to the methods described by Blank and Snyder (82) and Blank et al. (83). Additionally. differences in the positional specificity of the fatty acids will be determined by gas-liquid chromatography after treatment of phosphatidylcholine with phospholipase A_2 (84).

7. Priorities

The priorities of our proposed work will be essentially according to the order outlined (pp. 18-21). 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.

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 to 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.

Second year: Studies will begin utilizing alternate agents of induction (DMSO, retinoic acid) and morphological and biochemical markers for differentiation assessed. Phosphatidylcholine metabolism (^{32}P, [^{14}C]choline) will be investigated in control and induced cells (HL-60), and in TPA-resistant K562 leukemia cells. The optimal conditions for assaying choline kinase, phosphocholine cytidylyltransferase, and phosphocholinetransferase will be established and experiments initiated to determine the effects of TPA-induced differentiation on the activity of the 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. Throughout the 3-year period we will be examining plasma membrane, nuclear, and microsomal cell fractions in conjunction with our other experiments so that inductive locales (target sites) can be continually assessed.

1079629

E. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA

The biological materials used in this study consist of cultured cell lines of human origin that were established in 1977. Our cell culture facility is satisfactorily equipped to handle and dispose of this material in a safe fashion.

F. LABORATORY ANIMALS

None.

G. CONSULTANT

Dr. Fred Snyder of our Division has agreed to serve as a consultant in this research project. Also, within our Division we have an excellent cytogenetics department headed by Dr. Gayle Littlefield. She and her co-workers have agreed to aid in the cytologic aspects of morphological assessment of differentiation. Curriculum vita for Dr. Snyder and Dr. Littlefield are in Appendix III.

We have working arrangements with a number of scientists outside our group: cell biologists and toxicologists (Dr. Eliezer Huberman's group, Argonne 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 will be helpful in solving some of the specialized problems that might arise (cell differentiation, characterization, synthesis, and identification of compounds).

H. CONSORTIUM ARRANGEMENTS OR FORMALIZED COLLABORATIVE AGREEMENTS

None.

I. LITERATURE SITED

1. Baird, W. M. and Boutwell, R. K. (1971) Cancer Res. 31, 1074-1079.
2. Hecker, E. (1971) Methods Cancer Res. 6, 439-484.
3. Van Durren, B. L. (1969) Prog. Exp. Tumor Res. 11, 31-68.
4. Huberman, E. and Callaham, M. F. (1979) Proc. Natl. Acad. Sci. USA 76, 1293-1297.
5. Huberman, E., Heckman, C., and Langenbach, R. (1979) Cancer Res. 39, 2618-2624.
6. Snyder, F. and Wood, R. (1968) Cancer Res. 28, 972-978.
7. Snyder, F. and Wood, R. (1969) Cancer Res. 29, 251-257.
8. Berenblum, I. (1941) Cancer Res. 1, 44-48.
9. Hecker, E. (1966) Proc. IX Int. Cancer Congr., Tokyo (Abstracts. (p. 17).
10. Boutwell, R. K. (1978) In: Mechanisms of Tumor Promotion and Cacinogenesis (Slaga, T. J., Sivak, A., and Boutwell, R. K., eds.), Raven Press, New York, pp. 49-58.
11. Suss, R., Kreibich, G., and Kinel, V. (1972) Eur. J. Cancer 8, 299-304.
12. Yamasaki, H., Fibach, E., Hudel, U., Weinstein, I. B., Rifkind, R. A., and Marks, P. A. (1977) Proc. Natl. Acad. Sci. USA 74, 3451-3455.

1079630

13. Fibach, E., Gambari, R., Shaw, P. A., Maniatis, G., Reuben, R. C., Sassa, S., Rifkind, R. A., and Marks, P. A. (1979) Proc. Natl. Acad. Sci. USA 76, 1906-1910.
14. Rovera, G., O'Brien, T. G., and Diamond, L. (1977) Proc. Natl. Acad. Sci. USA 74, 2894-2898.
15. Ishii, D. N., Fibach, E., Yamasaki, H., and Weinstein, I. B. (1978) Science 200, 556-559.
16. Diamond, L., O'Brien, T. G., and Rovera, G. (1977) Nature (Lond.) 269, 247-249.
17. Cohen, R., Pacific, M. Rubenstein, N., Biehl, J., and Holtzer, M. (1977) Nature (Lond.) 266, 538-540.
18. Rovera, G., O'Brien, T. G., and Diamond, L. (1979) Science 204, 868-870.
19. Rohrschneider, L. R. and Boutwell, R. K. (1973) Cancer Res. 33, 1945-1952.
20. Balmain, A. and Hecker, E. (1974) Biochim. Biophys. Acta 362, 457-468.
21. Wertz, P. W. and Muller, G. C. (1978) Cancer Res. 38, 2900-2904.
22. Kinzel, V., Kreibich, G., Hecker, E., and Suss, R. (1979) Cancer Res. 2743-2750.
23. Castagna, M., Rochette-Egly, C., Rosenfeld, C., and Mishal, Z. (1979) FEBS Lett. 100, 62-66.
24. Dobbs, L. and Mason, R. J. (1978) Am. Rev. Respir. Dis. 118, 705-713.
25. Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-484.
26. Levine, L. and Ohuchi, K. (1978) Nature (Lond.) 276, 274-275
27. Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. (1980) Cancer Res. 40, 3674-3679.
28. Goldstein, I. M., Hoffstein, S. T., and Weissman, G. (1975) J. Cell Biol. 66, 647-652.
29. Biggar, W. D. (1978) Infect. Immun. 21, 669-671.
30. Friend, C., Scher, W., Hollond, J. G., and Soto, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378-382.
31. Ross, J., Ikawa, Y., and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3620-3623.
32. Ikawa, Y., Furasawa, M., and Sugano, H. (1973) Bibl. Haematol. 39, 955-967.
33. Collins, S. R., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458-2462.
34. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1979) J. Exp. Med. 149, 969-974.
35. Collins, S. J., Bodner, A., Ting, R., and Gallo, R. C. (1980) Int. J. Cancer 25, 213-218.
36. Breitman, T. R., Selonick, S. E., and Collins, S. J. (1980) Proc. Natl. Acad. Sci. USA 77, 2936-2940.
37. Wenner, C. E., Hackney, J., Kimelberg, H. K., and Mayhew, E. (1974) Cancer Res. 34, 1731-1737.
38. Lee, L-S. and Weinstein, I. B. (1978) J. Environ. Pathol. Toxicol. 1, 627-629.
39. Sivak, A., Mossman, B. T., and Van Duuren, B. L. (1972) Biochem. Biophys. Res. Commun. 46, 605-609.

1079631

40. Finean, J. B. (1973) In: Form and Function of Phospholipids, (Ansell, C. B., Hawthorne, J. N., and Dawson, R. M. C., eds.), Elsevier Scientific Publishing Co., Amsterdam, pp. 171-203.
41. Aizawa, Y. and Mueller, G. C. (1961) J. Biol. Chem. 236, 38-386.
42. Cunningham, D. D. (1972) J. Biol. Chem. 247, 2464-2470.
43. Fex, G. (1970) Biochem. J. 119, 743-747.
44. Pasternak, C. A. (1971) J. Cell Biol. 53, 231-234.
45. Spooner, P. M. and Gorski, J. (1972) Endocrinology 91, 1273-1283.
46. Kennedy, E. P. (1962) Harvey Lect. 57, 143-171.
47. Bremer, J. and Greenberg, D. M. (1961) Biochim. Biophys. Acta 46, 20-216.
48. Lands, W. E. M. (1960) J. Biol. Chem. 235, 2233-2237.
49. Lands, W. E. M. and Merkl, I. (1963) J. Biol. Chem. 238, 898-904.
50. Van Golde, L. M. G. and Van den Bergh, S. G. (1977) In: Lipid Metabolism in Mammals, Vol. 1, (Snyder, F., ed.), Plenum Press, New York, pp. 35-149.
51. Sundler, R., Arvison, G., and Akesson, B. (1972) Biochim. Biophys. Acta 280. 559-568.
52. Vance, D. E. and Paddon, H. B. (1978) Proc. Can. Fed. Biol. Soc. 21, 181.
53. Vance, D. E., Trip, E. M., and Paddon, H. B. (1980) J. Biol. Chem. 255. 1064-1069.
54. Kreibich, G., Hecker, E., Suss, R., and Kinzel, V. (1971) Naturwissenschaften 58, 323.
55. Cassileth, P. A., Suholet, D., and Cooper, R. A. (1981) Blood 58, 237-243.
56. Cooper, R. A., Ip, S. H. C., Cassileth, P. A., and Kuo, A. L. (1981) Cancer Res. 41. 1847-1852.
57. Suss, R., Kinzel, V., and Kreibich, G. (1971) Experimentia 27, 46-47.
58. Breitman, T. R., Collins, S. J., and Keene, B. R. (1980) Exp. Cell Res. 126, 494-498.
59. Vorbrodt, A., Meo, P., and Rovera, G. (1979) J. Cell Biol. 83, 300-307.
60. Manning, R. and Brindley, D. N. (1972) Biochem. J. 130, 1003-1012.
61. Rognstad, R., Clark, D. G., and Katz, J. (1974) Biochem. J. 140, 249-251.
62. Cabot, M. C. and Gatt, S. (1976) Biochim. Biophys. Acta 431, 105-115.
63. Weinhold, P. A. and Rethy, V. B. (1974) Biochemistry 13, 5135-5141.
64. Ansell, G. B. and Chojnacki, T. (1969) Methods Enzymol. 14. 121-125.
65. Weinhold, P. A., Skinner, R. S., and Sanders, R. D. (1973) Biochim. Biophys. Acta 326, 43-51.
66. Weiss, S. B., Smith, S. W., and Kennedy, E. P. (1958) J. Biol. Chem. 231, 53-64.
67. Vance, D. E. and Burke, D. C. (1974) Eur. J. Biochem. 43, 327-336.
68. Renkonen, O. (1966) Biochim. Biophys. Acta 125, 288-309.
69. Cabot, M. C. and Gatt, S. (1978) Biochim. Biophys. Acta 530, 508-512.
70. Koeffler, H. P., Bar-Eli, M., and Territo, M. D. (1981) Cancer Res. 41, 919-926.
71. Segal, A. W. (1974) Lancet 2. 1248-1251.
72. Ip, S. H. C. and Cooper, R. A. (1980) Blood 56, 227-232.
73. Depierre, J. W. and Karnovsky, M. L. (1973) Biochim. Biophys. Acta 320, 205-209.
74. Michell, R. H., Karnovsky, M. J., and Karnovsky, M. L. (1970) Biochem. J. 116, 207-216.
75. Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.

1079632

76. Cabot, M. C. and Snyder, F. (1980) Biochim. Biophys. Acta 617, 410-418.
77. Privett, O. S., Blank, M. L., Codding, D. W., and Nickell, E. E. (1965) J. Am. Oil Chem. Soc. 42, 381-393.
78. Snyder, F. and Kimble, H. (1965) Anal. Biochem. 11, 510-518.
79. Blank, M. L., Wykle, R. L., and Snyder, F. (1973) Biochim. Biophys. Acta 316, 28-34.
80. Rouser, G., Siakotos, A. N. and Fleischer, S. (1966) Lipids 1, 85-86.
81. Ways, P., Reed, C. F., and Hanahan, D. J. (1963) J. Clin. Invest. 42. 1248-1260.
82. Blank, M. L. and Snyder, F. (1970) Lipids 5, 337-341.
83. Blank, M. L., Lee, T-c., Piantadosi, C., Ishaq, K. S., and Snyder, F. (1976) Arch. Biochem. Biophys. 177, 317-322.
84. Cabot, M. C. and Snyder, F. (1978) Arch. Biochem. Biophys. 190, 838-846.

1079633

CHECKLIST

This is the required last page of the application.

Check the appropriate boxes and provide the information requested.

TYPE OF APPLICATION:

☐ NEW application *(This application is being submitted to the PHS for the first time.)*

☐ COMPETING CONTINUATION of grant number: _____.
(This application is to extend a grant beyond its original project period.)

☐ SUPPLEMENT to grant number: _____.
(This application is for additional funds during a funded project period.)

☐ REVISION of application number: _____.
(This application replaces a prior version of a new, competing continuation or supplemental application.)

☐ Change of Principal Investigator/Program Director.
Name of former Principal Investigator/Program Director: _____.

ASSURANCES IN CONNECTION WITH:

Civil Rights	Handicapped Individuals	Sex Discrimination	Human Subjects General Assurance *(If applicable)*	Laboratory Animals *(If applicable)*
[x] Filed	[x] Filed	[x] Filed	☐ Filed	[x] Filed
☐ Not filed	☐ Not filed	☐ Not filed	☐ Not filed	☐ Not filed

INDIRECT COSTS:

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHEW Regional Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHEW Guide for Establishing Indirect Cost Rates*, and submit it to the appropriate DHEW Regional Office. Indirect costs will not be paid on foreign grants, construction grants, and grants to individuals, and usually not on grants in support of conferences.

[x] DHEW Agreement Dated: 9 December 1980.

_____ % Salary and Wages or _____ % Total Direct Costs.

Is this an off-site or other special rate, or is more than one rate involved? ☐ YES [x] NO
Explanation: _____

☐ DHEW Agreement being negotiated with _____ Regional Office.

☐ No DHEW Agreement, but rate established with _____. Date _____.

☐ No Indirect Costs Requested.

1079634

Oak Ridge
Associated
Universities

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

Executive
Office

October 27, 1981

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. This grant is being submitted in place of an earlier submission by the same title and assigned NIH No. 1 R01 CA 29153-01. We request that the earlier application be withdrawn from consideration. The enclosed proposal has been re-written based on critical review of the earlier version. Bob Hawkins advised that Dr. Cabot request this substitution.

Oak Ridge Associated Universities is a nonprofit corporation sponsored by 51 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 on pages 10 and 11 of this 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
WILLIAM E. FELLING

William E. Felling
Acting Executive Director

br

Enclosures

cc: Dr. Thaddeus Domanski
Chemical and Physical Carcinogenesis Branch
Division of Cancer Cause and Prevention
National Cancer Institute
Bethesda, Maryland 20205

bcc: Dr. William R. Bibb DOE ORO (3) ←
Executive Office
W. F. Countiss
M. Cabot

1079635

Oak Ridge
Associated
Universities

Post Office Box
Oak Ridge, Tennessee
Telephone 615-

September 18, 1981

Dr. William R. Bibb, Director
Research Division
Department of Energy
Oak Ridge, TN 37830

Subject: DRAFT APPLICATION TO NIH FOR A PROJECT ENTITLED "LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA"

Dear Dr. Bibb:

Enclosed are three copies of a draft application to NIH for a project entitled "Lipid Metabolism in TPA-Differentiating Leukemia" to be carried out under the direction of Dr. Myles C. Cabot. If approved, this work 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 6-3122.

Formal copies of this grant application should be forwarded to NIH no later than October 26, 1981.

Sincerely,

Philip L. Johnson

Philip L. Johnson
Executive Director

RYAN:br

Enclosures

20.89.81

1079636

4 4253

FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH UCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT ☐ NO ☐ YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
CABOT, Myles C.

3b. SOCIAL SECURITY NUMBER
[redacted]

3c. MAILING ADDRESS (Street, city, state, zip code)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

3d. POSITION TITLE
Biochemist

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biological Chemistry

3f. TELEPHONE (Area code, number and extension)
615/576-3122

3g. MAJOR SUBDIVISION
Medical and Health Sciences Division

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
☒ NO ☐ YES (If "YES," form HEW 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
☒ NO ☐ YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: July 1, 1982 Through: June 30, 1985

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$220,000

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$69,400

9. PERFORMANCE SITES (Organizations and addresses)
Medical and Health Sciences Division
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
☒ NO ☐ YES - Previously reported
☐ YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, Tennessee 37830

Third Congressional District

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)
Code 2 0 Description:
(Medical and Health Sciences Division)

13. ENTITY IDENTIFICATION NUMBER
1620476816A1

14. TYPE OF ORGANIZATION (See instructions)
☒ Private Nonprofit
☐ Public (Specify Federal, State, Local):

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
William F. Countiss
Head, Office of Fiscal Services
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615/576-3056)

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
Philip L. Johnson
Executive Director
Oak Ridge Associated Universities
P. O. Box 117
Oak Ridge, TN 37830 (615/576-3300)

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)

DATE

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)

DATE

PHS-398
Rev. 10/79

1079637

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
LIC HEALTH SERVICE

ABSTRACT OF RESEARCH PLAN

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION (Same as Item 11, page 1)

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

TITLE OF APPLICATION (Same as Item 1, page 1)

LIPID METABOLISM IN TPA-DIFFERENTIATING LEUKEMIA

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

Myles C. Cabot, Biochemist, Biological Chemistry, Medical and Health Sciences Division

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

The potent tumor promoter, 12-0-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 as 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 phosphatidycholine 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 to supply information on the turnover of the hydrocarbon vs. polar head group moieties. The ultimate objective is to understand what factors are responsible for the TPA-induced stimulation of phosphatidycholine turnover in differentiating cells.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

SECTION 1. PAGE NUMBERS

Face Page, Abstract, Table of Contents 1-3
Detailed Budget for First 12 Month Budget Period 4
Budget Estimates for All Years of Support 5
Biographical Sketch-Principal Investigator/Program Director *(Not to exceed two pages)* ..... ____
Other Biographical Sketches *(Not to exceed two pages for each)* ..... ____
Other Support ..... ____
Resources and Environment ..... ____

SECTION 2.

Introduction *(Excess pages; revised and supplemental applications)* ..... ____
Research Plan
A. Specific Aims *(Not to exceed one page)* ..... ____
B. Significance *(Not to exceed three pages)* ..... ____
C. Progress Report/Preliminary Studies *(Not to exceed eight pages)* ..... ____
D. Methods ..... ____
E. Human Subjects, Derived Materials or Data ..... ____
F. Laboratory Animals ..... ____
G. Consultants ..... ____
H. Consortium Arrangements or Formalized Collaborative Agreements ..... ____
I. Literature Cited ..... ____
Checklist ..... ____

SECTION 3. Appendix *(Six sets) (No page numbering necessary for Appendix)*

Number of publications: _____ Number of manuscripts: _____
Other items (list):

Application Receipt Record, form PHS 3830
Form HEW 596 if Item 4, page 1, is checked "YES"

1079639

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: CABOT, Myles C.

DETAILED BUDGE. . OR FIRST 12 MONTH BUDGET PERIOD DIRECT COSTS ONLY

FROM 07/01/82 THROUGH 06/30/83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)		TIME/EFFORT				
NAME	TITLE OF POSITION	%	Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
Cabot, Myles C., Ph.D.	Principal Investigator	50	20	15,000	3,000	18,000
Welsh, Clement J.	Research Associate	50	20	9,430	2,120	11,350
TO BE NAMED	Postdoctoral or Research Technician	100	40	18,000	3,600	21,600
	SUBTOTALS →			42,430	8,720	51,150

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

Coulter Counter	$4,650	4,650

SUPPLIES (Itemize by category)

Tissue Culture Ware	$2,300	
Cell Culture Media & Serum	4,000	
Chemicals	1,700	
Radiolabeled Compounds	4,600	
		12,600

TRAVEL	DOMESTIC	1,000
	FOREIGN	
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

TOTAL DIRECT COSTS (Also enter on page 1, item 8) → $ 69,400

1079640

BUD T ESTIMATES FOR ALL YEARS OF 'PORT REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		51,150	56,300	61,900		
CONSULTANT COSTS						
EQUIPMENT		4,650	0	0		
SUPPLIES		12,600	13,900	15,300		
TRAVEL	DOMESTIC	1,000	1,000	1,000		
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES		0	500	500		
TOTAL DIRECT COSTS		$69,400	$71,700	$78,900		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 7) → $ 220,000

JUSTIFICATION (Use continuation pages if necessary): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

Recurring Increseas in Personnel Costs computed at 10%.

Equipment Justification:

1st Year - Coulter counter model ZF Serial 6600338 - Our present Coulter counter (model B) is an old, tube model with an excessive amount of down-time. Accurate, reproducible and rapid cell number assessment is an essential guideline for the current project, as ell number data will be used for differentiation criteria. The model ZF is the most economical counter of the series and offers all the features we require.

Personnel Justification:

Clement Welsh (Research Associate). Mr. Welsh was trained by me and works closely with me on the HL-60 project. He will be carrying out lipid composition analyses (These are time consuming and require skill). In addition, those duties which Mr. Welsh will aid include cell culturing, induction of differentiation protocol in vitro enzyme assay and laboratory management.

1079641

Postdoctoral or technician (to be named). This person will devote 100% of their time to project research; the position will require a working background in cell biology. The major duties of this position will be assessment of cell differentiation criteria (both morphological and biochemical) using the induction agents listed at various doses over various time intervals. In addition, in vitro enzyme assays of the phosphatidylcholine-metabolizing activities will be an important facet of this position.

1079642

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: CABOT, Myles C.

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Myles C. Cabot	Biochemist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

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

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

1976-1978: Damon Runyon-Walter Winchell Postdoc. Fellow in Cancer Research, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

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

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.

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, 838-846, 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 617, 410-418, 1980.

1079643

Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. *Alterations in lipid metabolism induced by 12-O-tetradecanoyl-phorbol-13-acetate in differentiating human myeloid leukemia cells.* Cancer Res. 40, 3674-3679, 1980.
Cancer Res. 40, 3674-3679, 1980.

Cabot, M. C. and Goucher, R.R. *Chaulmoogric acid: Assimilation into the complex lipids of mycobacteria.* Lipids 16, 146-148. 1981.

Cabot, M. C. and Lumb, R. H. *The activity of a low temperature lipase in the larvae of Sarcophaga bullata (Diptera: Sarcophagidae).* Comp. Biochem. Physiol. 68B, 325-328, 1981.

Cabot, M. D. and Welsh, C. J. *Ether lipid studies in mouse C3H/10T1/2 cells and a 3-methylcholanthrene transformed clone.* Arch. Biochem. Biophys. (in press).

Cabot, M. C. and Welsh, C. J. *Fatty acid metabolism in phorbol ester-differentiating human leukemia cells.* Cancer Res. (in press)

Cabot, M. C., Welsh, C. J., and Snyder F. *Modifying the levels of ether-linked lipids in L-M cells alters growth and choline utilization.* Exptl. Cell Res. (Submitted).

1079644

OTHER SUPPORT

(USE CONTINUATION PAGES IF NECESSARY)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications pending review and/or funding; (3) applications planned or being prepared for submission. Include all Federal, non-Federal, and institutional grant and contract support. If none, state "NONE." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent grant and the subproject and give the annual direct costs for each.) Briefly describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, justify and delineate the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) ACTIVE SUPPORT:

Investigator currently has no individual research support. He is supported under other larger projects:

A. American Cancer Society, Grant No. BC-70L; "Metabolism, Regulation and Function of Ether-Linked Glycerolipids and Their Precursors in Cancer Cells"; Dr. Fred Snyder: Senior Investigator; 20% Effort: $58,150 annual direct costs and entire period support; Purpose is to study the metabolism and regulation of ether- and ester-type glycerolipids in selected cancer cells.

B. NIH; Grant No. 5 R01 CA 11949-12; "Ether Lipids in Cancer - Enzyme Mechanisms"; Dr. Fred Snyder: Senior Investigator; 25% effort; $57,404 annual direct costs; $305,031 TDC entire period of support; Purpose is to purify key enzymes involved in the metabolism of ether linked lipids, so their kinetic properties and reaction mechanisms can be established.

C. DOE; Contract No. DE-AC05-760R00033; "Lung Surfactant, Membrane, Lipids and Energy Biohazards"; Dr. Fred Snyder: Senior Investigator; 55% effort; $223,549 annual direct costs and entire support period; Purpose of research focuses on health problems created by newer energy technologies; study the effect of chemical toxicants, carcinogens and radiation on systems being investigated.

(2) NONE.

(3) NIH; Grant Application; "Lipid Metabolism and Differentiators of Leukemia"; Dr. Myles Cabot; Senior Investigator; 50% effort; $64,500 1st year annual direct costs; $376,500 TDC total support period; The purpose to establish alterations in cellular lipid composition between undifferentiated cells and cells treated with various inducers of differentiation.

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe facilities at other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include a description of the nature of any collaboration with other organizations and provide further information in the RESEARCH PLAN.

[X] Laboratory: My laboratory is approximately 17 x 25 ft.; it is qell equipped for carring out experiments and has two offices adjacent, one of which contains a freezer and refrigerator. The lab is located in the biochemistry department of the Medical and Health Sciences Division with analytical and preparative capacities at hand.

[ ] Clinical:

[ ] Animal:

[X] Computer: We have immediate access to computer ware.

[X] Office: My office is located adjacent to my laboratory.

[X] Other (Tissue culture): We have two culture facilities, on e located directly across the hall from my laboratory. The human cell culture lab is located upstains (approx. 45 seconds away).

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location, and pertinent capabilities of each. Lipid nitrogen refrigerator; thin-layer chromatographic equipment, including zonal scraper and spark chamber; photodensitometer; preparative and analytical centrifuges; Model E ultracentrifuge, liquid scintillation spectrometer, gas-liquid chromatographs; HPLC; Beckman DU and Acta C-III spectrometer; Cahn electrobalance; protein isolation equipment. All the equipment is in excellent working order and located in the labs adjacent to my laboratory. Service contracts are active.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

We have on board secretarial staff, machine and electronics shop, access to electron microscopy, mass spectrometry; consultants in the areas of cell biology, cytogenetics, organic chemistry. The support services are readily available.

1079646

DETACH AND CLIP TO THE SIGNED FACE PAGE OF THE APPLICATION

PERSONAL DATA ON

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitoring the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director.

To provide the PHS with the information it needs for this important task, the principal investigator/program director is requested to complete the form below and attach a single copy to the signed face page of the application.

Upon receipt and assignment of the application by the PHS, this form will be detached from the application. It will NOT be duplicated and will NOT be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant Contract Information)." All analyses conducted on the data will report aggregate statistical findings only and will not identify individuals.

If you decline to provide this information, it will in no way affect consideration of your application.

Your cooperation will be appreciated.

Date of Birth: [redacted] (Month/Day/Year)

Sex: ☐ Female ☒ Male

Race and/or Ethnic Origin:

Check one:

- ☐ American Indian or Alaskan Native
- ☐ Asian or Pacific Islander
- ☐ Black, not of Hispanic origin
- ☐ Hispanic
- ☒ White, not of Hispanic origin

NOTE: The category that most closely reflects the individual's recognition in the community should be used for purposes of reporting mixed racial and/or ethnic origins. Definitions are on the back of form.

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 (polar head group, hydrocarbon moieties) 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. We endeavor to determine if phosphatidylcholine turnover is linked to the induction of differentiation. This query can in part be answered by comparing ^{32}P and ^{14}C choline utilization in cells induced to differentiate by treatment with dimethyl sulfoxide and retinoic acid. Finally we wish to define the location of the induction by TPA leading to the lipid alterations by examining the assimiliation of labeled precursors in isolated subcellular fractions.

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

1019648

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 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). We have recently shown (Cabot et al., 1980) that TPA-induced differentiation of human myeloid leukemia cells (HL-60) is accompanied by a stimulation of phospholipid metabolism (before the markers of differentiation are expressed), enhanced incorporation of acetate into free fatty acids and neutral lipids, an increase in the amount of cellular triacylglycerols, and a selective incorporation of fatty alcohol into triacylglycerols and their ether-containing analog, alkyldiacylglycerols.

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 morphologial 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

1019649

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).

Although the action of TPA as an inducer of cell differentiation has been the object of considerable study, a variety of compounds, unrelated chemically, have been shown to induce morphological and biochemical alterations in numerous cell lines. DMSO induces erythroid differentiation in mouse erythroleukemia (Friend) cells and the morphological, biochemical and immunological changes induced have been well characterized (Friend et al., 1971; Ross et al., 1972; Ikawa et al., 1973). The differentiation-inducing effect of DMSO has recently been demonstrated in human cells (Collins et al., 1978; Huberman et al., 1979; Collins et al., 1979; Collins et al., 1980), and retinoic acid, another patent differentiator has been shown to induce maximal differentiation (Approx. 90%) of human promyelocytic leukemia cells at a concentration of 1μM, and amount much less than the concentration needed for DMSO to produce similar differentiation (Breitman et al., 1980b). These studies suggest that HL-60 cells share common target sites for the induction of differentiation by these compounds.

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 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. Additionally, a shortcoming of previous studies has been the failure to define if the stimulation of phosphatidylcholine metabolism is linked to the induction of differentiation in HL-60 cells. Although TPA has been shown to significantly alter phosphatidylcholine metabolism in HL-60 cells and other cell lines, DMSO and retinoic acid, effective agents of induction, have not been investigated. Kinzel et al., (1979) have shown that DMSO enhances the incorporation of [^{3}H] choline into HeLa cells. DMSO was not as effective as TPA, however these investigators used 0.5%, an amount equal to that which is introduced when TPA is added to cultures using DMSO as a vehicle, but not sufficient to induce maximal differentiation of HL-60 cells. (Collins et al., 1978).

Phosphatidylcholine, a structural component of animal cells, has been implicated in the modulation of certain enzyme activities (Finean, 1973). Phospholipid metabolism has also been linked to events leading to the stimulation of growth (Aizawa and Mueller, 1961; Cunningham, 1972; Fex, 1970; Pasternak, 1972; Spooner and Gorski, 1972). 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

1079650

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. Our investigations as well as studies by others, demonstrate that one of the earliest events elicited by TPA is the stimulation of phosphatidylcholine metabolism. This information reinforces the theory that membranes are an important target of phorbol esters, and thus could modulate signals which initiate cellular responses.

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 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

Our initial results which document dramatic alterations in lipid metabolism occuring in TPA-differentiated human myeloid leukemia cells have been published (Cabot et al., 1980). The data and description of these experiments can be seen in the attached reprint (Appendix I). Briefly, this work shows that TPA-induced differentiation of HL-60 cells is accompanied by a stimulation of phospholipid metabolism before the markers of differentiation are expressed. Phospholipid metabolism was assessed by differences in the utilization of labeled precursors, ^{32}p and [^{14}C]choline, by control and TPA-treated cells. Although other changes in lipid metabolism were demonstrated, we beleive, from our most recent studies (manuscript, Appendix I) that increased triacylglycerol levels documented in treated cells is a characteristic inherent to the differentiated cell, whereas the flux in phosphatidylcholine metabolism is early in the event of differentiation. This may suggest that the stimulation of phosphatidylcholine metabolism by TPA is linked to the induction of differentiation.

The effects of TPA-induced differentiation on phospholipid metabolism have been limited to studies using labeled precursors of the polar head groups, thus little is known about the effects of phorbol ester-induction on fatty acid metabolism. A topic of ongoing investigation has therefore been in the direction of acyl group metabolism of the phospholipids (manuscript, Appendix I). As the data and description of these experiments are outlined in detail, only a brief description of that investigation follows. In cells incubated with labeled fatty acids for 1 hr, the percent distribution of lipid radioactivity is highest in the phospholipids of undifferentiated cells, whereas in TPA-supplemented cultures, substantially more label is associated with triacylglycerols. This trend was confirmed by data derived from analysis of lipids in undifferentiated and TPA-treated cells (8×10^{-10}M TPA, 48 hr), where in the latter, the amount of triacylglycerols increased 3.2-fold. The increase in the amount of cellular phospholipids was not as pronounced. Whereas a marked stimulation of the incorporation of ^{32}p and choline into phosphatidylcholine has been shown to occur in TPA-differentiated cells, acyl group metabolism in phospholipids is affected to a much lesser degree. Thus the turnover of the polar portion of the molecule appears to be stimulated more than the hydrophobic moieties of phospholipids. An interesting aside to fatty acid metabolism in differentiating cells was the finding that the desaturation of stearic acid (conversion of [1-^{14}C]stearic acid to [1-^{14}C]oleic acid by cell cultures) was greatly reduced compared to control activity. A corrolary reflecting the cellular consequences of this phenomenon was shown by the marked differences in acyl group composition of triacylglycerols in differentiating cells, which contained decreased amounts of unsaturates compared to the triacylglycerol fatty acid profile of undifferentiated cells.

We have endeavored to determine if the rate of uptake of free fatty acids is higher in differentiated cells. At early times after TPA exposure (1 - 4 hr) there was no stimulation of the cellular uptake of labeled fatty acids, however differentiated cells (24-48hr TPA-treated), when exposed to labeled acids, contained more radioactivity than undifferentiated cells. This phenomenon was however variable and dependent on the fatty acid supplemented, with arachidonic acid showing the greatest uptake differential. When [1-^{14}C]palmitic acid and TPA were added simultaneously to cultures and incubated for 48 hr, the cells contained approximately twice the radioactivity (dpm/mg cell protein) compared to cells labeled in the absence of TPA for the same time period.

1079652

Qualitative analyses of the lipids in the control and TPA-treated cells showed the latter to contain a 4-fold increase in the amount of triacylglycerol-associated radioactivity.

The study of acyl group metabolism in phospholipids of differentiated cells has only been touched on; we have confirmed that the phosphatidylcholine fraction isolated from TPA-differentiated cells, labeled with [1-^{14}C]stearic acid, contains more radioactivity than the ethanolamine-containing phosphoglycerides (Approx. 2 times). Our preliminary data on fatty acid metabolism coupled with our previous findings on polar head group utilization has yielded significant information to form a natural backdrop for continued studies. We are most excited with our results of late: by utilizing the culture system described by Brietman et al., (1980a), we now have HL-60 cells (passage 27) growing in the absence of serum. The serum required to grow cells is a rich source of fatty acids. It has always been an advantage for one interested in lipid studies to have a versitile system wherein cells can be grown either in the presence or absence of serum. Furthermore, we have just demonstrated that these cells, grown in the absence of serum, can be induced to differentiate into a highly enriched population of macrophage-like cells (this is the first report of this phenomenon). This is a significant finding, in view of the fact that serum-grown cells treated with TPA have been morphologically characterized as both macrophage-like and granulocytic, even though they possess high acid phosphatase levels (Vorbrodt et al., 1979), an enzymatic marker for macrophages. The acyl group composition of phospholipids and triacylglycerols from cells grown with serum and in a serum-free medium is shown in Table I (Appendix II). Compared to the fatty acid composition of serum-grown cells, the serum-free cells demonstrate a relatively simple acyl profile; most notably is the near absence of polyunsaturates with 16:0 + 16:1 + 18:1 comprising about 80% of the total species, and as shown by the DMA derivatives, plasmalogen levels are 5-fold lower. In view of the fact that HL-60 cells can be passaged in the absence of serum and differentiate in the presence of TPA, this system will be extremely useful in studies aimed at acyl group metabolism of phosphatidylcholine and ether lipid modulations in differentiation.

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.

D. METHODS OF PROCEDURE

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

The modification of phosphatidylcholine metabolism is the first lipid-related event 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, inositol- and serine-containing phosphoglycerides, triacylglycerols,

1079653

alkyldiacylglycerols, and cholesterol. These experiments will in turn be carried out with cells grown in serum-free media. Additional, 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. Ether phosphoglyceride levels will also be extensively analyzed in isolated plasma membrane and nuclear fractions. The general protocol for lipid compositional analyses during induced differentiation will involve growing the cells in media containing TPA (8×10^{-10} - 1.6×10^{-8}M) for periods up to 3 days. The extracted lipids (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.

Radiolabeled palmitic, steric, oleic, and arachidonic acid will be either added to the growth medium (plus serum, serum-free) 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 48 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. Purified plasma membrane, microsomal, and nuclear fractions will additionally be examined.

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 inttial 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 concentration of TPA in the incubation medium to more thoroughly assess the effects of TPA on transport and metabolic fate of the labeled precursor. Because it is important to define whether the stimulated phosphatidylcholine turnover observed during TPA induction is linked to differentiation, we will carefully examine ^{32}P and [^{14}C]choline uptake and utilization in cells induced with dimethylsulfoxide and retinoic acid. Transport of labeled choline will be measured by the disappearance of radioactivity from the cell media. The rate of 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 little 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 fatty acids into cellular phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. Acyl group turnover will be assessed by labeling a series of control and TPA-treated cultures 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.

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, differentiation, and subcellular fractionation

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 pennicillin (100 units/ml) and streptomycin (100 μg/ml). Cell numbers will be determined from cell counts after excluding cells strained with trypan blue. Cells will be treated with TPA 24 hr after subculture. The concentration of TPA in the culture medium will be 8×10^{-10}M. This concentration in our hands has been sufficient to induce differentiation, however experiments will be conducted using a range of concentrations, 10^{-9} - 1.6×10^{-8}, the highest being the amount employed by most investigators. Explicit details of cell culture procedure, TPA supplementation and evaluation of cell differentiation have been cited elsewhere (Huberman and Callaham, 1979; Cabot et al., 1980). TPA will be added to culture media in DMSO so that its final concentration does not exceed 0.01%. This amount of DMSO has no effect on cell growth or differentiation. Terminal differentiation induced by other agents (Collins et al., 1978) will be carried out by incubating cells for 6 days in 1.25% DMSO, in a like manner for cells grown in serum-free media (Breitman et al., 1980), or in media containing retinoic acid (1×10^{-6}M) as described by Breitman et al., (1980b).
We have shown in our previous study that the stimulation of phosphatidylcholine metabolism preceeds the induction of differentiation markers (Cabot et al., 1980). 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, inducer concentration, and degree 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. Cellular differentiation (morphological) will be assessed periodically by performing differential counts on Wright-stained cell preparations and based on the percentage of myelocytes, metamyelocytes and banded and segmented neutrophils. The control population consists primarily of promyelocytes (90%). Other criteria include cell attachment and ceasation of growth. Macrophage populations will also be assessed. Biochemical markers for differentiation include NBT dye reduction (Segel, 1974; Collins et al., 1980), phagocytosis of Candida albicans (Collins et al., 1978), lysozyme release (Biggar, 1978), and specifically in the case of TPA, acid phosphatase activity for the monocytic macrophage differentiation produced (Vorbrodt et al., 1979).

Plasma membranes will be isolated following a published protocol specific for HL-60 cells (Ip and Cooper, 1980). Nuclei will be isolated according to a procedure described for guinea pig polymorphonuclear leukocytes (Depierre and Karnovsky, 1973). This procedure employs the homogenization of cells in a medium that is slightly hypotonic followed by immediate restoration of isotonicity. By this method the integrity of subcellular organelles is maintained. The purity of the subcellular fractions will be established by assay of classical marker enzymes. We will additionally utilize the methods of De Pierre and Karnovsky (1973) and Michell et al., (1970) which provide invaluable techniques for the isolation and characterization of subcellular fractions.

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).

1019651

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). Additionally 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. 12-17). 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.

We have a number of collaborative ties with scientists outside our group: cell biologists and toxicologists (Dr. Eliezer Huberman's group, Argonne 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). Within our Division we have an excellent cytogenetics department headed by Dr. Gayle Littlefield. She and her co-workers have agreed to aid in the cytologic aspects of morphological assessment of differentiation.

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 III).

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.

Second year: Studies will begin utilizing alternate induction agents (DMSO, retinoic acid) and morphological and biochemical markers for differentiation assessed. Phosphatidylcholine metabolism (^{32}P, [^{14}C]choline) will be investigated in control and induced cells. The optimal conditions for assaying choline

1019658

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. Throughout the 3-year period we will be examining plasma membrane, nuclear, and microsomal cell fractions in conjunction with our other experiments so that inductive locals (target sites) can be continually assessed.

1079659

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
Bigger, W. D. (1978) Infect. Immun. 21, 669
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
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
Breitman, T. R., Collins, S. J., and Keene, B. R. (1980) Exp. Cell Res. 126, 494
Breitman, T. R., Selonick, S.E., Collins, S. J. (1980) Proc. Natl. Acad. Sci. USA 77, 494
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 617, 410
Cabot, M. C., Welsh, C. J., Callaham, M. F., and Huberman, E. (1980) Cancer Res. 40, 3674
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
Collins, S. J., Bodner, A., Ting, R., and Gallo, R. C. (1980) Int. Jr. Cancer, 25, 213.
Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458.
Cunningham, D. D. (1972) J. Biol. Chem. 247, 2464
DePierre, J. W. and Karnovsky, M. L. (1973) Biochim. Biophys. Acta 320, 205
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
Friend, C., Scher, W., Hollond, J. G., and Soto, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378
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
Ikawa, Y., Furasawa, M., and Sugaro, H. (1973) Bibl Haematol. 39, 955
Ip, S. H. C., and Cooper, R. A. (1980) Blood 56, 227

1079660

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
Michell, R. H., Karnovsky, M. J., and Karnovsky, M. L. (1970) Biochem. J. 116, 207
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
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
Ross, J., Ikawa, Y., and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3620
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
Segal, A. W. (1974) The Lancet, 2, 1248.
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
Vorbrodt, A., Meo, P., Rovera, G. (1979) J. Cell Biol. 83, 300
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

1079661

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, and liquid nitrogen refrigerator. We have also recently established a laboratory specially designed for human cell culture. 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 ^{3}H 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), a Cahn electrobalance (Model 25), and high pressure liquid chromatographs. We also have access to electron microscopy.

'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

1079662

Privileged Communication

C. I, Myles C.
[redacted]

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

1079663