

Miami Winter Symposia

Volume 12

CANCER ENZYMOLOGY

Edited by

J. SCHULTZ

F. AHMAD

MIAMI WINTER SYMPOSIA - VOLUME 12

Cancer Enzymology

**edited by
J. Schultz
F. Ahmad**

PAPANICOLAOU CANCER RESEARCH INSTITUTE,
MIAMI, FLORIDA



*Proceedings of the Miami Winter Symposia, January 1976
Sponsored by The Papanicolaou Cancer Research Institute
Miami, Florida*



Academic Press, Inc. New York San Francisco London 1976

A Subsidiary of Harcourt Brace Jovanovich, Publishers

COPYRIGHT © 1976, BY ACADEMIC PRESS, INC.

ALL RIGHTS RESERVED.

NO PART OF THIS PUBLICATION MAY BE REPRODUCED OR TRANSMITTED IN ANY FORM OR BY ANY MEANS, ELECTRONIC OR MECHANICAL, INCLUDING PHOTOCOPY, RECORDING, OR ANY INFORMATION STORAGE AND RETRIEVAL SYSTEM, WITHOUT PERMISSION IN WRITING FROM THE PUBLISHER.

ACADEMIC PRESS, INC.

111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by

ACADEMIC PRESS, INC. (LONDON) LTD.

24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 76-20852

ISBN 0-12-632745-9

PRINTED IN THE UNITED STATES OF AMERICA

SPEAKERS, CHAIRMEN, AND DISCUSSANTS

- F. Ahmad**, Papanicolaou Cancer Research Institute, Miami, Florida.
- M. Bade**, Boston College, Chestnut Hill, Massachusetts.
- R.E. Block**, Papanicolaou Cancer Research Institute, Miami, Florida.
- Z. Brada**, Papanicolaou Cancer Research Institute, Miami, Florida.
- A.M. Brown**, National Cancer Institute, Rockville, Maryland.
- J.M. Buchanan** (Session Chairman), Massachusetts Institute of Technology, Cambridge, Massachusetts.
- J.J. Byrnes**, University of Miami School of Medicine, Miami, Florida.
- B.F. Cameron**, Papanicolaou Cancer Research Institute, Miami, Florida.
- B. Chance** (Session Chairman), University of Pennsylvania, Philadelphia, Pennsylvania.
- Z.F. Chmielewicz**, State University of New York, Buffalo, New York.
- R.W. Estabrook** (Session Chairman), University of Texas, Dallas, Texas.
- D.A. Goldthwait**, Case Western Reserve University, Cleveland, Ohio.
- S.B. Greer**, University of Miami, Miami, Florida.
- S. Grossman**, Union Carbide Corporation, Tarrytown, New Jersey.
- J.E. Harrison**, Papanicolaou Cancer Research Institute, Miami, Florida.
- M. Horowitz**, New York Medical College, Mt. Vernon, New York.
- F. Huijing**, University of Miami, Miami, Florida.
- W. Jakoby**, National Institutes of Health, Bethesda, Maryland.

SPEAKERS, CHAIRMEN, AND DISCUSSANTS

N.O. Kaplan (Session Chairman), University of California, San Diego, California.

H. Kareem, University of Miami, Miami, Florida.

S. Klebanoff, University of Washington, Seattle, Washington.

G. Koch, Roche Institute of Molecular Biology, Nutley, New Jersey.

R. Leif, Papanicolaou Cancer Research Institute, Miami, Florida.

F. Lynen, Max-Planck-Institute für Biochemie, Bei Munchen.

S. Magnusson, University of Aarhus, Aarhus.

J. McCord, Duke University, Durham, North Carolina.

L. Menahan, Medical College of Wisconsin, Milwaukee, Wisconsin.

A. Mildvan (Session Chairman), Institute for Cancer Research, Philadelphia, Pennsylvania.

H.P. Morris, Howard University, Washington, D.C.

R.E. Parks, Brown University, Providence, Rhode Island.

H. Petering, University of Cincinnati, Cincinnati, Ohio.

M. Rouleau, National Institutes of Health, Bethesda, Maryland.

J. Schultz, Papanicolaou Cancer Research Institute, Miami, Florida.

A.H. T. Theorell, Karolinska Institutet, Stockholm, Sweden.

B.L. Vallee (Session Chairman), Harvard University, Boston, Massachusetts.

J. Van Lancker, University of California, Los Angeles, California,

G. Weber (Session Chairman), Indiana University, Indianapolis, Indiana.

S. Weinhouse (Session Chairman), Temple University, Philadelphia, Pennsylvania.

A.A. Yunis, University of Miami School of Medicine, Miami, Florida.

PREFACE

In recent years a great deal of progress has been made on the covalent modifications of proteins and how they affect physiological function. One of these modifications that profoundly affects many cellular functions involves proteolysis. Therefore, the main theme of the Eighth Miami Winter Symposia arranged by the Department of Biological Chemistry, University of Miami, concerns "Proteolysis and Physiological Regulation." As in the past, the program arranged by the Papanicolaou Cancer Research Institute is complimentary to this basic theme. This year we chose "Cancer Enzymology."

The selection of Dr. Hugo Theorell as the Lynen Lecturer made it possible for us also to select old friends and students of Dr. Theorell: namely, John Buchanan and Britton Chance. It also brought back to the symposia previous participants such as Sidney Weinhouse, Ronald Estabrook, George Weber, and Seymour Klebanoff. The plan of the program started with the strategy of the cell and progressed to bioenergetics, regulation and cytotoxicity, thus providing a role for enzymes in the mechanism of the living as well as protection against adverse environment.

It has always been a pleasure to meet with those who have attended the symposia in the past and continue to do so, each time exercising and expressing their pleasure at the format and general atmosphere of this Gordon Conference-like meeting taking place in mid-winter. The restful atmosphere in Miami at this time of year is conducive to more relaxed discussion and interaction among the participants. Such interaction is not readily possible at large national meetings where both the subject matter and the number of participants limits the opportunity to meet informally and enjoy the criticism of objective observers. It is, therefore, our sincere hope that future Miami Winter Symposia will be attended by many former participants, as well as many new scientists, to share in a mutually stimulating week of academic exchange.

We would like to acknowledge the support of Eli Lilly and Company, Abbott Laboratories, Hoffman-LaRoche, Inc., MC/B Manufacturing Chemists, and the Upjohn Company. Finally, it is of particular importance that the recording of the discussions and the preparation of this volume was made possible through the combined efforts of our expert typists, Kathi Bishop, Bonnie Tracy, and Anne Johnson, who worked under the direction of Mrs. Ginny Salisbury.

*J. Schultz
F. Ahmad*

CONTENTS

SPEAKERS, CHAIRMEN AND DISCUSSANTS
PREFACE

ix
xi

Protease-Related Effects in Normal and Transformed Cells 1

J.M. Buchanan, L. Bo Chen, and B.R. Zetter

DISCUSSION: *Magnusson*

The Secretion of Fibrinolysin by Cultured Mammalian Tumor Cells 25

M.C. Wu, D.R. Schultz, and A.A. Yunis

DISCUSSION: *Brown, Buchanan, and Magnusson*

Isozyme Composition, Gene Regulation, and Metabolism
of Experimental Hepatomas. 41

S. Weinhouse, J.B. Shatton, and H.P. Morris

DISCUSSION: *Chance, Kaplan, Parks, Horowitz,
Grossman, and Huijing*

Enzymatic Strategy of the Cancer Cell. 63

G. Weber

DISCUSSION: *Menahan, Grossman, Morris, Koch
Parks, Chance, and Buchanan*

Oxygen Reduction by Cytochrome Oxidase — A Possible
Source of Carcinogenic Radical Intermediates 89

B. Chance

DISCUSSION: *Weber, Theorell, Vallee, Estabrook
Kaplan, Kareem, and Mildvan*

The Activation of Polycyclic Hydrocarbons:
Cytochrome's P-450, Oxygen and Electrons. 103

R.W. Estabrook, V.W. Patrizi, and R. Prough

DISCUSSION: *Chance, Weinhouse, Mildvan,
Vallee, Parks, and Jakoby*

CONTENTS

Magnetic Resonance Studies of the Mechanism of DNA Polymerase I from <i>E. Coli</i>	123
<i>A.S. Mildvan, D.L. Sloan, C.F. Springgate, and L.A. Loeb</i>	
DISCUSSION: <i>Vallee, Estabrook, Greer, Parks, and Block</i>	
Enzymes Involved in Repair of DNA Damaged by Chemical Carcinogens and γ -Irradiation	139
<i>D.M. Kirtikar, J.P. Kuebler, A. Dipple, and D.A. Goldthwait</i>	
DISCUSSION: <i>Van Lancker, Grossman, and Greer</i>	
Zinc Biochemistry in the Normal and Neoplastic Growth Processes.	159
<i>B.L. Vallee</i>	
DISCUSSION: <i>Brada, Mildvan, Weinhouse, Schultz, Bade, Petering, and Chmielewicz</i>	
Target Directed Cancer Chemotherapeutical Agents.	201
<i>N.O. Kaplan</i>	
DISCUSSION: <i>Leif, Weinhouse, Menahan, and Parks</i>	
Regulation of Fatty Acid Biosynthesis in Mammary Tumors	229
<i>F. Ahmad, P. Ahmad, and D. Schildknecht</i>	
DISCUSSION: <i>Lynen, Weinhouse, Cameron, and Rouleau</i>	
Selective Inhibition of the 3' to 5' Exonuclease Activity Associated with Mammalian DNA Polymerase δ	245
<i>J.J. Byrnes, K.M. Downey, V. Black, L. Esserman, and A.G. So</i>	
DISCUSSION: <i>Mildvan and Koch</i>	
Myeloperoxidase-Mediated Cytotoxicity	267
<i>S.J. Klebanoff, R.A. Clark, and H. Rosen</i>	
DISCUSSION: <i>Schultz, Mildvan, Leif, and Estabrook</i>	
Cytotoxicity of the Superoxide Free Radical	289
<i>J.M. McCord and M.L. Salin</i>	
DISCUSSION: <i>Harrison, Estabrook, and Horowitz</i>	
The Functional Mechanism of Myeloperoxidase	305
<i>J. Harrison</i>	
DISCUSSION: <i>Weinhouse and Klebanoff</i>	
Myeloperoxidase-Enzyme Therapy on Rat Mammary Tumors.	319
<i>J. Schultz, A. Baker, and B. Tucker</i>	
DISCUSSION: <i>Mildvan and Leif</i>	

CONTENTS

Free Communications

Defective Regulation of Cholesterol Biosynthesis in Tumor-Virus Transformed and Hypercholesterolemic Human Skin Fibroblasts: A Comparative Study	335
<i>J.M. Bailey, T. Allan, E.J. Butler and J.D. Wu</i>	
Viral Stimulation of Choline Phosphotransferase in Spleen Microsomes During Production of Malignancy	336
<i>W.E. Cornatzer, D.R. Hoffman and D. Skurdal</i>	
L-Asparaginase with Antilymphoma Activity From <i>Vibrio succinogenes</i>	337
<i>J.A. Distasio and R.A. Niederman</i>	
Malate-Aspartate Shuttle Activity in Several Ascites Tumor Lines	338
<i>W.V.V. Greenhouse and A.L. Lehninger</i>	
Inhibition by Serum of Intracellular Degradation of Human Chorionic Gonadotropin (hCG)	339
<i>R.O. Husa and R.A. Pattillo</i>	
The Interaction of Antitumor Drugs with Folate Requiring Enzymes	340
<i>D.W. Jayme, P.M. Kumar, N.A. Rao, J.A. North, and J.H. Mangum</i>	
Studies on the Collagenolytic Activity of Methylcholanthrene- Induced Fibrosarcomas in Mice	341
<i>K.R. Labrosse and I.E. Liener</i>	
Activities of Enzymes of Glycolysis and Adenine Nucleotide Metabolism During Murine Leukemogenesis	342
<i>L.A. Menahan and R.G. Kemp</i>	
The Association of a Protease (Plasminogen Activator) with a Specific Membrane Fraction Isolated From Transformed Cells	343
<i>J.P. Quigley</i>	
Structural Features of <i>S. Typhimurium</i> Lipopolysaccharide (LPS) Required for Activation of Monocyte Tissue Factor	344
<i>F.R. Rickles and P.D. Rick</i>	
Role of Plasminogen Activator in Generation of MIF-Like Activity by SV3T3 Cells	345
<i>R.O. Roblin, M.E. Hammond, P.H. Black, and H.F. Dvorak</i>	
Proteolysis and Cyclic AMP Levels in Cell Culture	346
<i>W.L. Ryan, M.L. Heidrick, and G.L. Curtis</i>	

CONTENTS

Specificity of DNA-Dependent RNA Polymerase Activities in Rabbit Bone Marrow Erythroid Cell Nuclei	347
<i>M.K. Song and J.A. Hunt</i>	

PROTEASE-RELATED EFFECTS IN NORMAL AND TRANSFORMED CELLS

JOHN M. BUCHANAN, LAN BO CHEN AND BRUCE R. ZETTER
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

INTRODUCTION

The basic problem of cell biology concerned with the mechanism of stimulation of resting or dormant cells to proliferation has been approached in several ways. There is now a relatively long list of compounds that have been shown to be mitogenic for certain cell systems. This list includes serum (1-3), the protein hormones (4), insulin and insulin-like materials, such as multiplication stimulating activity (5) and somatomedin (6), fibroblast growth factor (7) and epidermal growth factor (8). Other biological materials including fetuin (9), seromucoid (10), lipopolysaccharide (11), glucocorticoids (12) and lectins (11) have also been reported to be mitogenic.

A third group of mitogenic substances, the proteases, have been of particular interest to us since the observation by Sefton and Rubin (13) that trypsin stimulates resting chick embryo cells to division and the report by Burger (14) in the same year that trypsin, ficin, and pronase can stimulate the growth of density-inhibited 3T3 cells. At the time of initiation of studies in our laboratory the role of proteases as mitogens had not been generally explored in many cell systems.

The use of proteases as mitogens seemed particularly attractive initially because they had been reported to react with proteins on the cell surface causing specific alterations that have been proposed to have special significance in the basic mechanism of the processes leading to cell division (15,16). Specifically, a cell surface protein of 250,000 daltons that can be enzymatically iodinated is removed by trypsin (17-20) and is absent from the external surface of many transformed cells (17-25). Furthermore, a protease capable of activating plasminogen has been indirectly implicated in maintaining the phenotype

of transformed cells (26-30). Thus, proteases have been proposed as active agents in the metabolism of both normal and transformed cells.

At the onset of our experiments we felt that certain restrictions should be applied to the choice of a protease selected for study.

It should

- 1) be a major constituent of the circulating fluids normally found in blood, and therefore in plasma, as a proenzyme, which can be activated if the need for cell proliferation occurs,
- 2) be readily available in relatively large quantities in highly purified form,
- 3) exhibit a high degree of proteolytic specificity,
- 4) serve as a mitogen for a host cell that will respond to this enzyme when present alone without the requirement of any other survival, attachment or growth factors.

Of the several possible choices among the kallikreins and the proteases of the clotting and complement systems, thrombin was selected as most nearly fulfilling these qualifications. Its proenzyme form, prothrombin, is present in blood at a level of 100 γ per ml. Serum contains active thrombin since the latter is produced in excess of the capacity of antithrombin proteins to neutralize it (31). The relationship of thrombin to other blood proteases is shown in Fig. 1.

Thrombin is a highly specific proteolytic enzyme that is known to split, for example, 4 arginyl-glycine bonds of fibrinogen (32) and a few selected lysyl and arginyl bonds of actin (33). It has been supplied to us in highly pure form from bovine and human sources by Dr. David F. Waugh and Dr. John W. Fenton, respectively.

RESULTS

Thrombin as a mitogen:

When added as a single component to the culture medium

of chick embryo fibroblasts, thrombin is capable of stimulating these cells to division (34). The assay for mitogenicity consists of measuring the response of resting cells for DNA synthesis from labeled thymidine 12 hr after addition of the reagent or after 4 days for total cell count.

By both criteria thrombin was highly mitogenic. When compared to graded levels of serum ranging from 0 to 5 percent, thrombin was equally effective when added between 0 and 10 μg per ml. Prothrombin, itself, was inactive but when incubated in a Ca^{++} -containing medium to which Factor X_a and V had been added in very small quantities, the proenzyme was converted to the active agent, thrombin. In this conversion the chick cells themselves provided the thromboplastin, a colloidal lipoprotein necessary for this reaction (see Fig. 1).

We then explored the relative proportions of thrombin-yielding components of defibrinogenated plasma to other mitogenic activities as calibrated by use of the chick embryo fibroblast as the host cell. The answer to this question was first approached by determination of the inhibition of the mitogenic activity of thromboplastin-treated plasma with phenylmethylsulfonylfluoride, which combines with and inactivates the "serine" proteases (32). Approximately 50% of the mitogenic activity of plasma was lost by this procedure. However, treated-plasma may contain other "serine" proteases with mitogenic activity, but in far less quantities than thrombin. One example is Factor X_a , which has been shown in this laboratory to be mitogenic (unpublished results).

Therefore, in order to assess the true contribution of prothrombin in plasma we separated the proteins in defibrinogenated plasma that are absorbed to BaSO_4 and tested the mitogenic activities of both the non-absorbed (Fraction I) and absorbed proteins (Fraction II) before and after treatment with thromboplastin. It was found that the total mitogenic activity of Fraction I did not depend on treatment of the preparation with thromboplastin, but that the activity of Fraction II was greatly enhanced by this treatment. When chromatography was performed on either the untreated or treated samples of Fraction II, we could show that in the former case the mitogenic activity was eluted with prothrombin and in the latter instance with thrombin. The mitogenic activity from thrombin precursors of plasma

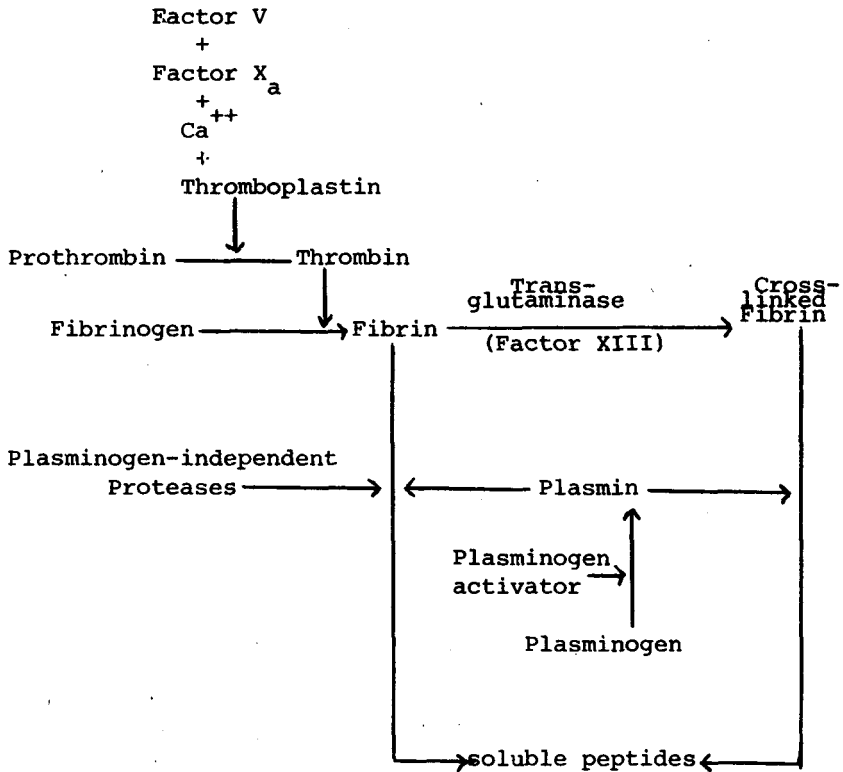


Fig. 1. Enzyme systems involved in the formation and dissolution of fibrin clots.

is approximately 30 percent of the total mitogenic activity. Thus, by either method of analysis, i.e. by specific analysis or by enzymatic inhibition with phenylmethyl-sulfonylfluoride, the thrombin-yielding material of plasma represents an important share of the mitogenic activity of this fluid. Therefore, from a quantitative point of view thrombin formed from prothrombin might be expected to play a role under certain physiological conditions, for example in wound healing. The concentration of thrombin in the microenvironment of the wound during clot formation probably

is considerably greater than that of collected serum and would be sufficient to initiate cell proliferation in the wound area, a necessary second step in restoring the damaged area to normal condition.

Since these experiments were conducted entirely with secondary cultures of chick embryo fibroblasts, we then examined the role of thrombin in other cell systems. When added to several murine embryo fibroblasts (mouse-Swiss, mouse BalB C, Rat, and Golden Hamster) thrombin was also strongly mitogenic. In a very surprising manner it could not under these conditions cause cell proliferation when tested on chick embryo fibroblasts transformed with Rous sarcoma virus. Whether this lack of response to thrombin represents a requirement for other serum factors or whether the transformed cell simply could not respond to the protease as a growth stimulator required further investigation.

There are certain cell lines that are unresponsive to thrombin when the latter is present as the sole mitogen, for example, 3T3, 3T6, CHO, BHK and Nil. In recent experiments we have examined the effects of thrombin on some of these cells when it is added in combination with other mitogenic factors.

In Fig. 2 we show results of an experiment with a mouse BalB C, 3T3 fibroblast cell line infected with Avian sarcoma virus B77. As the accessory factors we selected Cohen's epidermal growth factor (8) and prostaglandin F2 α . The latter has been demonstrated to have a stimulatory effect on DNA synthesis and cell proliferation of Swiss 3T3 cells (35). The B77-3T3 line cell was used in these experiments because we have found that it is particularly responsive to epidermal growth factor. The cell count in all cases was normalized to the growth response obtained with 5% calf serum.

At a low concentration of serum (0.7%) B77-3T3 is maintained in culture but exhibits only minimal growth. When provided with thrombin at a level of 0.2 μ g/ml there was no growth beyond this basic level. Highly purified epidermal growth factor supplied at a level of 1.5 ng/ml evinces a response 1.4 times that of the 5% serum level. At a level of 0.6 ng/ml the response is only 50%. It is at this latter concentration that effects of other factors can be demonstrated. For example, addition of prostaglandin F2 α at

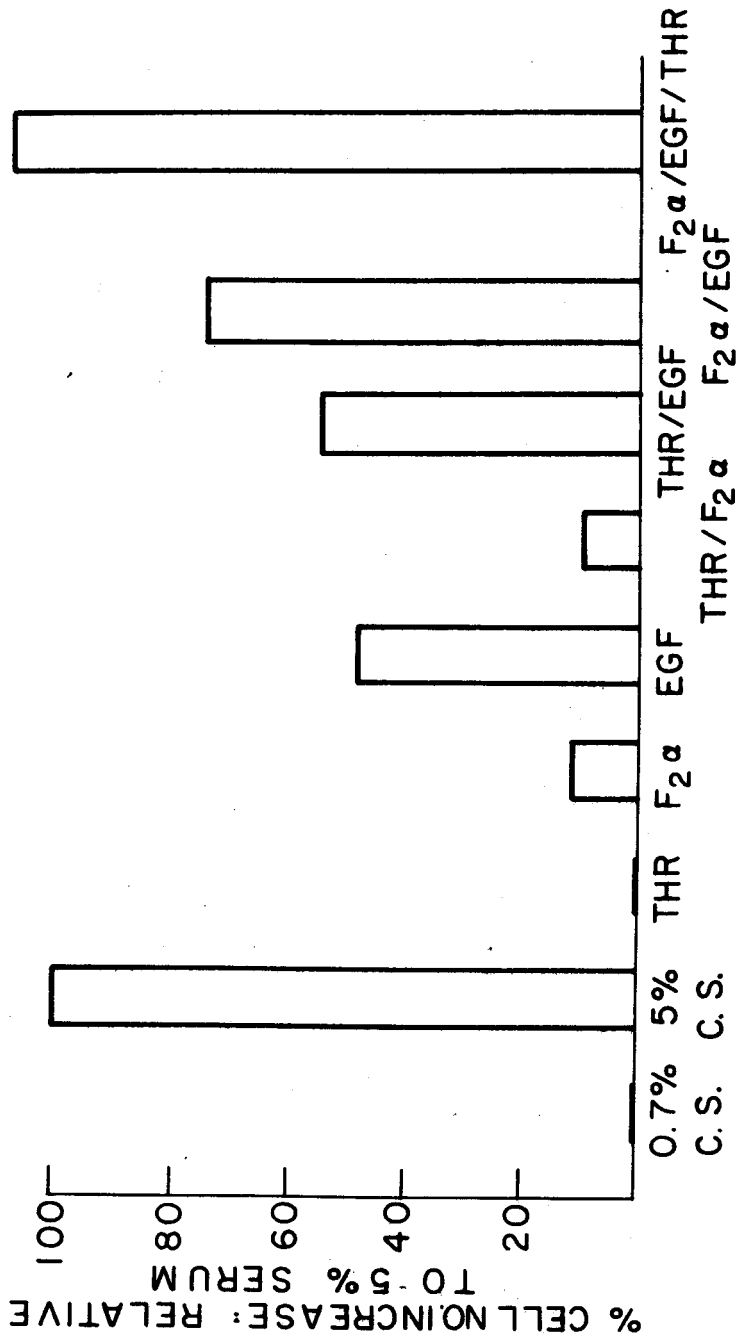


Fig. 2. Potentiation of cell growth of B77-3T3 mouse fibroblasts by thrombin in the presence of other co-mitogens. EGF=epidermal growth factor (0.6 ng/ml) Thr=thrombin (0.2 μ g/ml), F2 α =prostaglandin F2 α (0.2 μ g/ml), C.S. = calf serum.

a concentration of 0.2 μ g/ml to the B77-3T3 cells results in an increase of only 10% of that of 5% serum. Combination of thrombin with either F2 α or EGF does not yield a further stimulation of cell division. However, combinations of F2 α and EGF show slightly more than the additive effects of either alone, but in the presence of all three factors, F2 α , EGF and thrombin, a response greater than that evoked with 5% serum is obtained. Therefore, it is possible to demonstrate a role for thrombin provided that other essential factors are present. This potentiation for thrombin cannot be demonstrated, however, at the higher saturating concentrations of EGF. Similar synergistic effects of EGF and arginine esterase (or thrombin) on the stimulation of DNA synthesis in an explant of human foreskin has been independently observed by Lembach (36).

The Role of Surface Proteins in Cell Proliferation as Studied with Thrombin and Other Proteases:

As stated in the introductory comments, special attention has centered on an iodinated cell surface protein of 250,000 daltons (250K) of chick embryo fibroblasts, since it is removed by trypsin during mitogenic stimulation and is lost by some transformed cells. We have attempted to provide further evidence about the role of this protein in mitogenesis by use of thrombin (37). Thrombin is ideally suited for this type of investigation because of its mitogenicity and because of its great specificity as demonstrated by the limited number of peptide bonds it can split. In addition to trypsin and thrombin, several other proteolytic enzymes including bromelin, ficin, chymotrypsin, subtilisin, pronase, and α -protease, were tested both for their capacity to stimulate cell proliferation (or DNA synthesis) and to cleave iodinated cell surface proteins of chick embryo fibroblasts. Our first efforts centered around an attempt to establish a correlation between mitogenicity and cell surface composition after protease treatment. Two comparisons stood out as particularly revealing, namely, that thrombin could stimulate cells to divide, yet did not cleave the 250K