

University of New Hampshire

University of New Hampshire Scholars' Repository

Doctoral Dissertations

Student Scholarship

Fall 1972

THE EFFECTS OF DIBUTYRYL CYCLIC 3',5'-ADENOSINE-MONOPHOSPHATE AND RELATED COMPOUNDS ON (TRITIUM-LABELED) THYMIDINE AND (CARBON-14-LABELED) LEUCINE INCORPORATION BY NEWT LIMB REGENERATION BLASTEMAS IN VITRO

GEORGE LEON BABICH

Follow this and additional works at: <https://scholars.unh.edu/dissertation>

Recommended Citation

BABICH, GEORGE LEON, "THE EFFECTS OF DIBUTYRYL CYCLIC 3',5'-ADENOSINE-MONOPHOSPHATE AND RELATED COMPOUNDS ON (TRITIUM-LABELED) THYMIDINE AND (CARBON-14-LABELED) LEUCINE INCORPORATION BY NEWT LIMB REGENERATION BLASTEMAS IN VITRO" (1972). *Doctoral Dissertations*. 998.

<https://scholars.unh.edu/dissertation/998>

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact Scholarly.Communication@unh.edu.

INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.
2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.
3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.
4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106
A Xerox Education Company

73-4359

BABICH, George Leon, 1944-
THE EFFECTS OF DIBUTYRYL CYCLIC 3',
5'-ADENOSINE MONOPHOSPHATE AND RELATED
COMPOUNDS ON ^3H -THYMIDINE AND ^{14}C -LEUCINE
INCORPORATION BY NEWT LIMB REGENERATION
BLASTEMAS IN VITRO.

University of New Hampshire, Ph.D., 1972
Zoology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THE EFFECTS OF DIBUTYRYL CYCLIC 3', 5'-ADENOSINE MONOPHOSPHATE AND
RELATED COMPOUNDS ON ^3H -THYMIDINE AND ^{14}C -LEUCINE INCORPORATION BY
NEWT LIMB REGENERATION BLASTEMAS IN VITRO.

by

GEORGE L. BABICH

B.A., Atlantic Union College, 1966
M.S., University of New Hampshire, 1969

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Zoology

September, 1972

This thesis has been examined and approved.

John E. Foret

Thesis director, John E. Foret, Asst. Prof. of Zoology

Paul A. Wright

Paul A. Wright, Prof. of Zoology

Samuel C. Smith

Samuel C. Smith, Assoc. Prof. of Biochemistry and Poultry Science

Edward K. Tillinghast

Edward K. Tillinghast, Assoc. Prof. of Zoology

James A. Stewart

James A. Stewart, Asst. Prof. of Biochemistry

Aug. 14, 1972

Date

PLEASE NOTE:

Some pages may have
indistinct print.

Filmed as received.

University Microfilms, A Xerox Education Company

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all those who have helped in the completion of this dissertation.

Special gratitude is due the members of my committee for their suggestions in the preparation of this manuscript.

Additional appreciation is expressed to Dr. J.E. Foret, committee chairman, for his help in microscopic and photographic techniques as well as his everyday guidance and encouragement in completing this project. Appreciation is also expressed to Dr. Tillinghast who along with Dr. Foret grasped the significance of cyclic AMP and instilled it into his students.

This investigation was supported in part by National Defense Education Act, Title IV. Fellowships (67-06745.1 and 68-02742.1) and a grant from the Biomedical Sciences (#NIH-5505-FR-07108) Committee of the University of New Hampshire.

For the background in zoology and the academic environment provided by the Department of Zoology at the University of New Hampshire, the author is deeply thankful.

TABLE OF CONTENTS

LIST OF FIGURES.....	iv
LIST OF TABLES.....	v
ABSTRACT.....	vi
I. INTRODUCTION.....	1
II. MATERIALS AND METHODS.....	10
III. RESULTS.....	17
1. Studies on Protein Synthesis.....	17
a. Relationship between dibutyryl cyclic AMP and protein synthesis in early and late blastemas...	17
b. The effects of dibutyryl cyclic AMP and NaF.....	17
c. The effects of agents which alter phosphodies- terase activity.....	21
d. Cyclic AMP and NaF effects on denervated limbs..	21
2. Studies on DNA Synthesis.....	24
a. Effects of dibutyryl cyclic AMP and NaF.....	24
b. Effects of agents which alter phosphodiesterase activity.....	24
c. The effects of dibutyryl cyclic AMP on dener- vated blastemas.....	24
3. The Effects of Acetylcholine on ³ H-thymidine and ¹⁴ C-leucine Incorporation.....	28
IV. DISCUSSION.....	31
1. Studies on Protein Synthesis.....	31
2. Studies on DNA Synthesis.....	35
3. The Effects of Acetylcholine on ³ H-thymidine and ¹⁴ C-leucine Incorporation.....	37
BIBLIOGRAPHY.....	38
APPENDIX.....	45

LIST OF FIGURES

Number		Page
1.	Section through the regenerating early mound stage blastema (100 X).....	11
2.	26 day blastema explanted onto a Millipore filter strip.....	12
3.	Explanted blastema on a Millipore filter strip in a Falcon Plastics culture dish.....	14
4.	Dosage Study. Effects of various concentrations of dibutyryl cyclic AMP on ^{14}C -leucine incorporation by 14-16 day limb regenerates.....	18
5.	Dosage Study. Effects of various concentrations of dibutyryl cyclic AMP on ^{14}C -leucine incorporation by 26-28 day limb regenerates.....	19
6.	Effect of dibutyryl cyclic AMP and NaF on ^{14}C -leucine incorporation by early and late regenerates denervated 72 hours prior to explantation.....	23
7.	Effect of dibutyryl cyclic AMP and NaF on ^3H -thymidine incorporation by early and late regenerates denervated 72 hours prior to explantation...	27
8.	Effect of acetylcholine on ^{14}C -leucine and ^3H -thymidine incorporation by early and late limb regenerates.....	29
9.	14 day blastema explanted onto a Millipore filter strip and grown in the presence of acetylcholine chloride (900 X).....	30

LIST OF TABLES

Number		Page
1.	The effects of various agents on ¹⁴ C-leucine incorporation by cultured amphibian forelimb regeneration blastemas.....	20
2.	The effects of some agents on ³ H-thymidine incorporation by cultured amphibian forelimb regeneration blastemas.....	25

ABSTRACT

The effects of dibutyryl cyclic 3', 5'-adenosine monophosphate and related compounds on ^3H -thymidine and ^{14}C -leucine incorporation by newt limb regeneration blastemas in vitro.

By

George L. Babich

1. An organ culture system is described as a convenient method for studying the effects of cyclic AMP and compounds related to its metabolism on protein synthesis by newt forelimb regeneration blastemas. Preparations of limb blastemas grown in a modified Lebowitz L-15 medium exhibit similar biochemical responses to cyclic AMP as have been reported in other biological systems. A neurochemical has been implicated in the partial maintenance of protein synthesis in the regenerating limb, normally a nerve dependent system. During the early mound stage, a time of critical neural dependency, supplementing the explanted, hence denervated, limb blastema with exogenous dibutyryl cyclic AMP or sodium fluoride significantly enhances leucine incorporation over the level observed in contralateral controls from the same animal. In older palette regenerates, a stage of suspected neural independence, the dibutyryl cyclic AMP-induced incorporation of leucine is somewhat less than that observed in the mound stage but still about double the control value. Sodium fluoride, a stimulator of adenylate cyclase, increases the rate of leucine incorporation at the later stage as well. During extended denervations (72 hours before explantation) mound stage blastemas cultured in the presence of exogenous dibutyryl cyclic AMP also exhibit increased levels of leucine incorporation. Sodium fluoride, on the other

hand, caused a decrease in protein synthesis in older palette stage blastemas which had been denervated prior to explantation. This could conceivably be interpreted as due to a time-dependent loss of adenylate cyclase activity. Theophylline, imidazole, and cyclic phosphodiesterase, all reported to affect the level of cyclic AMP in other systems, were ineffective in altering leucine incorporation by cultured blastemas.

2. The effects of dibutyryl cyclic AMP and other agents related to cyclic AMP metabolism on DNA synthesis by cultured newt limb regeneration blastemas were studied. Dibutyryl cyclic AMP stimulated thymidine incorporation in both early and late stages of regeneration, although the effect was more pronounced in the latter. It also increased DNA synthesis in regenerates which had been maintained nerveless for 72 hours prior to explantation, although the effect was significant only in the later stage of regeneration. Theophylline significantly increased thymidine uptake by early regenerates, whereas sodium fluoride was effective in the later stage only, and had no significant effect on blastemas previously denervated. Neither imidazole nor cyclic AMP-phosphodiesterase had a significant effect in the concentrations used.

3. Cultured amphibian limb blastemas at both the early and late stages of regeneration were treated with acetylcholine. Incorporation of radioactive leucine or thymidine was measured after 24 hours of incubation. A significant reduction in leucine incorporation was observed in experimental cultures, particularly at the earlier stages of regeneration, whereas

DNA synthesis was apparently unaffected. It is suggested that acetylcholine may be involved in the regulation of protein synthesis during regeneration of the newt limb in vivo.

4. The data obtained from the above experiments were interpreted to support the hypothesis implicating the adenylate cyclase-cyclic AMP system in amphibian limb regeneration.

INTRODUCTION

Limb regeneration in urodele amphibians requires the accumulation of mesodermally-derived cells into a regeneration blastema. Following amputation, lysis of the internal stump tissues releases mesenchymal cells, which accumulate at the tip of the stump and below the apical epidermal cap (Butler and O'Brien, 1942; Hay, and Fischman, 1961). The dedifferentiated cells proliferate, then later redifferentiate to form a functionally competent limb (Thornton, 1938; Schmidt, 1968).

Neural, epidermal, and hormonal tissues have been shown to influence significantly the processes of regeneration in the limb (Schotte' and Butler, 1941; Rose, 1944; Singer, 1952; Thornton, 1960, 1970). Schotte' and Butler (1944) observed that the presence of nerve tissue is required throughout the mound stage of regeneration. Singer and Craven (1948) demonstrated that denervation 17 days post-amputation (ie. between the mound and the cone stage) inhibited the proliferation of blastemal cells in the adult newt. It was Singer (1952) who later concluded that the type of nerve was unimportant, but that the essential neural requirement was a threshold ratio of nerve fiber diameter to amputation surface area. This observation was later supported by Rzehak and Singer (1966). Dresden (1969) proposed that nerve tissue may be intimately involved in the control of protein, DNA and RNA synthesis in the regenerate. Lebowitz and Singer (1970) observed that a particular fraction of spinal nerve homogenate was responsible for forty per cent of the nerve directed protein synthesis in the early blastema (12-14 days post-amputation), at a time when the suspected neuro-

trophic factor exerts much of its influence. The proposed neurotrophic factor which stimulates regeneration has not been identified but is thought to be of high molecular weight (Deck and Futch, 1969). Singer and Inoue (1964) observed that the nerves of the stump rapidly enter the overlying epithelium becoming intimately associated with it. Motor fibers do not enter the wound epithelium (Singer, 1959; Thornton, 1960), however, a limb stump innervated only by motor fibers can regenerate (Singer, 1946). The observation of neural invasion of the epidermis of the regenerate suggested that nerve infiltration may reflect an interaction between nerve and epidermal tissue. The same group, along with others, reported that there is a continual distal flow of materials toward the apical epithelial cap, which may indicate a possible mode of information transport from limb stump to epidermal cells. Because of the reports of distal information flow by Becker (1972), and those of Singer and Inoue (1964) reporting intimate contact between nerve fibers and epithelial cells, one can propose an information exchange system based on the proximity of the cells involved. Recently, Carlson (1969) and Dresden (1969) reported that interruption of either innervation or of apical cap metabolism will disrupt the normal redifferentiation of blastemal cells.

In addition to innervation, limb regeneration is normally dependent upon the presence of an overlying apical cap. Stevens et al. (1965) emphasized that blastemal aggregation occurs only when there is intimate contact between the thickened apical epithelium of the limb and the mesodermal cells inferior to it. Both nerve fibers and apical cap cells presumably secrete substances that are trophic in nature. The identity of these trophic substances is currently the object

of investigation by many groups but is yet unknown. Thornton (1965) proposed that the apical cap dictates the proper geometry and spatial relationships of the advancing, redifferentiating tissues.

Tassava (1969) reported that the endocrine system influences the continued development and maintenance of the newt limb regenerate. Other studies have shown that interruption of the adrenocortico-hypothalamic-pituitary axis will inhibit limb regeneration in the adult newt (Schotte' and Hall, 1952). In addition, Schotte' (1961) reported that the stress of amputation triggers the release of ACTH from the pituitary gland which mediates the release of adrenal hormones. In the absence of these hormones there is no blastemal development and regeneration is aborted. Hypophysectomy of adult urodeles has been shown to decrease the level of RNA in the tissues of the amputated limb (DeConinck et al., 1956). This decrease in RNA level could be attributed to the decrease in STH (Talwar et al., 1965). Future studies must now be directed towards re-investigating the influence of hormones on limb regeneration in view of recent advances in biochemistry and endocrinology. Recent reports reviewed by Hardman et al., (1971) indicate that in many biological systems, hormonal and humoral activity is consistently related to the intracellular levels of adenosine 3', 5'-monophosphate (cyclic AMP). Since many hormonal actions involve increased or decreased levels of cyclic AMP, the latter compound seems to be involved in a generalized control mechanism for many of the systems thus far investigated. As a result Robison, Butcher, and Sutherland (1968) proposed that cyclic AMP acts as a "second messenger" in hormone action. According to this concept, hormones or other factors can be termed first messengers. After their release from the cells of origin

and transport to the target cells these interact with the membrane-bound enzyme, adenylate cyclase. Rall and Sutherland (1962) reported that adenylate cyclase catalyzes the formation of cyclic AMP from ATP. This interaction between the first messenger and the adenylate cyclase system results in an altered level of intracellular cyclic AMP, the "second messenger", which may affect a number of cellular processes. The exact relationship between cellular receptors and adenylate cyclase is still unknown (Hardman, 1971).

Sutherland, Rall, and Menon (1962) noted that cyclic AMP as well as adenylate cyclase are found in relatively high concentration in the central nervous system, especially at synaptic junctions (DeRobertis, et al., 1967). These compounds may also be associated with the neuromuscular junction (Goldberg and Singer, 1970). Since limb regeneration is normally a nerve dependent process, cyclic AMP might be involved in limb regeneration. Furthermore, Konijn (1970) demonstrated the presence of cyclic AMP in early amphibian embryos, thus suggesting the possibility that the compound might be associated with a variety of developmental processes. Foret (personal communication) has recently obtained evidence of relatively high levels of adenylate cyclase activity in regenerating newt limbs.

Cyclic AMP is now recognized as the mediator of a variety of hormonal effects. Activation or inactivation of adenylate cyclase by the first messenger results in drastic alterations in cyclic AMP levels within the cell (Sutherland, et al., 1965). In addition, cyclic AMP levels can be altered by affecting its rate of degradation. Cyclic AMP is rapidly degraded to non-cyclic 5'-AMP by one or more cellular phosphodiesterases which are specific for 3', 5'-mononucleotides (Butcher and Sutherland, 1962). This enzymatic hydrolysis is the only

reported mechanism for inactivating cyclic AMP. The phosphodiesterases are inhibited by a number of agents, including methylxanthines (e.g. theophylline), certain antibiotics, papaverine, and a number of unrelated substances as well. Cheung (1966) reported enzyme inhibition with ATP. Butcher and Sutherland (1962) reported that imidazole stimulates the activity of phosphodiesterase thus lowering the level of cyclic AMP. Phosphodiesterase inhibitors which are able to penetrate into cells have been found to be potent lipolytic agents (Vaughan and Steinberg, 1963; Butcher et al., 1965). Another method of increasing intracellular levels of cyclic AMP is by exposing cells to exogenous cyclic AMP or derivatives of the compound. Dibutyryl cyclic AMP (N^6 , O^2' -dibutyryl adenosine 3', 5'-cyclic monophosphate) is routinely used in some laboratories because it readily penetrates cell membranes (Posternak et al., 1962; Rosen et al., 1970). This attempt to control the level of cyclic AMP is considered drastic since nucleotides are primarily intracellular compounds. However, in certain experimental situations it has been possible to mimic the effects of first messengers or phosphodiesterase inhibitors by using physiological concentrations of cyclic AMP, ranging from 10^{-2} to 10^{-7} molar (Butcher et al., 1965). In addition, Meester (1967) and Murad et al. (1962) inhibited adenylate cyclase with choline esters and caffeine. Recently, Van DeVeerdonk (1971) inhibited with acetylcholine the cyclic AMP-dependent effects of MSH on melanocytes in Xenopus laevis.

Foret (personal communication) presented evidence suggesting a possible role of cyclic AMP in amphibian limb regeneration. This proposal is compatible with the work of Konijn (1970) who demonstrated the presence of cyclic AMP in amphibian gastrulas, an interesting

observation because of the similarities between ontogeny and limb regeneration. In view of the presence of cyclic AMP in amphibians, and the nerve-dependent process of limb regeneration as well as the possible implications of cyclic AMP in neural function, we thought it worth-while to study what effects, if any, dibutyryl cyclic AMP or related agents might have on the metabolism of the isolated limb regenerate.

An approach to determining the nature of the neural influence in regeneration was introduced by Singer (1954) who utilized the micro-infusion of microquantities of test substances into the intact regenerating limb. This approach provides a relatively constant exposure of specific target tissues to the test agent without resorting to the in toto immersion of the animal or injection of the test compounds into the entire circulatory system. Organ culture would seem preferable, however, to the above approaches, because the concentration of test substances at the target organ can be more critically controlled. Technical difficulties of the organ culture procedure were surmounted by Stocum (1968) and later modified by Dresden (1969) and Sooy (1970). Isolating the blastema in vitro precludes limb stump influences and permits a replacement type of experimental design. Thus the blastema can be cultured at a time of neural dependency, while replacing the neural influences with the proposed neurotrophic factor(s).

In this study, the Trowell grid technique (Trowell, 1959) is used. The blastema is oriented so that the mesodermally-derived cells are exposed to the medium and to the additives. This type of blastema orientation on a Millipore filter allows the test agent and nutrient to pass through the tissues thus approximating the pre-explantation

environment. The early blastema is used to test a stage which is clearly nerve dependent (Schotte' and Butler, 1944; Singer and Craven, 1948; Chalkley, 1954; Powell, 1969). The later paddleform blastema was used to investigate the role of cyclic AMP during a stage of suspected neural independence and high mitotic activity (Hay and Fischman, 1961).

Singer and Craven (1948), Chalkley (1954) and Rzehak and Singer, (1966) observed that the early phases of regeneration are characterized by increased protein and nucleic acid synthesis, both requiring a threshold level of neural tissue to maintain this level of synthesis. Bodemer and Everett (1959) reported that protein synthesis is augmented in the vicinity of the cut brachial nerve and suggested that this phenomenon is related to a neurotrophic effect. Overton (1950) demonstrated that neural tissue transplanted beneath amphibian skin will stimulate mitotic activity. Hay and Fischman (1961), studying changes in DNA activity in the blastema, observed that in the early blastema (10-15 days post-amputation) the apical cap cells cease to synthesize DNA. The cap increases in size primarily because the limb epithelium proximal to the amputation surface is actively synthesizing DNA, proliferating, and supplying cells to the apical cap. Since these apical cap cells were not synthesizing DNA, Hay and Fischman (1961) speculated that these cells may be involved in some specific function in regeneration. With the earlier information on nerve and apical cap dependency, and the data of Singer and Salpeter (1960) and Hay and Fischman (1961) on the close relationship between blastema cells with nerve and the apical cap, it is possible to propose an interaction between apical cap cells, blastema cells, and some unknown mediator which controls blastemal cells' behavior. Cyclic AMP

has been implicated as the mediator of various regulatory activities in other physiological systems (Greengard and Costa, 1970). Cyclic nucleotides appear to control the early steps in a series of events which activate protein kinases (Walsh, 1968; Kuo, 1969; Soldering, 1970; Gill, 1970; Klein, 1971) and may be involved in nucleic acid metabolism (Malamud, 1969). Therefore, the two parameters that were used in this study as criteria for possible cyclic AMP interaction in the regenerating limb were changes in the level of ^{14}C -leucine and ^3H -thymidine incorporation.

The nervous system is involved in growth and the maintenance of structural integrity, in addition to the function of information transmission. Singer (1954) was first to suggest the possibility that nerve fibers emit trophic chemicals from their distal endings. In a nerve-dependent system such as the regenerating limb, the neurotrophic theory has been tested at both the morphological level (Butler and Schotte', 1949; Schotte' and Butler, 1944; Singer, 1949; Bryant, 1970) and the biochemical level (Lebowitz and Singer, 1970; Dresden, 1969). Rall (1969) examining the action of cyclic AMP on humoral transmission, reported that cyclic AMP alters the level of synaptic activity by acting as an intracellular messenger for humoral agents. Recent evidence of Goldberg and Singer (1970) clearly showed that cyclic AMP is the intermediate in a system that controls the release of acetylcholine at the neuromuscular junction. Krnjevic and Meledi (1958) reported that epinephrine, which is controlled by cyclic AMP, mediates the release of acetylcholine released by nerve-muscle and sympathetic ganglion preparations. Singer et al. (1960) found that agents which

inhibit the action of acetylcholine suppress regeneration. Later, the results were held in question by the authors. Murad (1962) and Meester et al., (1967) reported that choline esters will depress adenylate cyclase activity, thus decreasing the ambient level of cyclic AMP. It has been suggested by Katz (1966) that acetylcholine interacts with receptor molecules at the post-synaptic membrane, altering it so that it becomes more permeable to small cations, and, therefore, decreasing the membrane resistance. Rasmussen (1970) reviewed the reports of numerous contractile and secretory phenomena and proposed a close relationship between the calcium ion and cyclic AMP activity in the cell membrane. This was later followed by a report by McLennan (1970) which demonstrated that the calcium ion is essential for the release of acetylcholine from nerve tissue.

The primary object of this study was to analyze the effects of denervation and cyclic AMP replacement on protein and DNA synthesis in the regeneration blastema grown in vitro. The index of the agents' effect on regeneration is the alteration in ^{14}C -leucine or ^3H -thymidine incorporation, which presumably is correlated to changes in protein or DNA synthesis. The results suggest that in the early bud and paddleform stage, there is a close relationship between protein and DNA synthesis and cyclic AMP levels. The results therefore support the hypothesis that the cyclic AMP-adenylate cyclase system is involved in amphibian limb regeneration, and is perhaps related to the neurotrophic factor emitted by nerve endings within the regeneration blastema.

Materials and Methods

Adult newts, Notophthalmus (= Triturus) viridescens, were obtained from local supply houses and from ponds in the vicinity of Durham, New Hampshire. The animals were reared in large aquaria containing dechlorinated tap water. The newts were fed and the water changed twice weekly. Regenerating animals were kept at 20°C.

Blastema preparation:

Both forelimbs were amputated through the distal third of the humerus with fine iridectomy scissors, thus providing a flat surface from which the regenerated portion of the limb was later removed. The animals were maintained post-operatively in distilled water without antibiotics. Two stages of blastemal development were studied. The early mound stage regenerate (14-16 days at 20°C) consists of a low mound of accumulated mesenchyme-like cells covered by a thickened apical epithelial cap (Fig. 1.). The late or palette stage of regeneration (26-28 days at 20°C), a phase of high mitotic activity just before cytodifferentiation, consists of a flattened distal growth (Fig. 2.). If the regeneration plane was not clearly defined, differences in limb pigmentation served as a rough guideline for blastemal removal.

Preparation of the blastemal explant:

At the appropriate stage the regenerates were excised after a 24-hour period in which the animals were immersed in distilled water containing penicillin (0.4gm/l), streptomycin (10^5 units/l), and mycostatin (10^3 units/l). The limbs were removed at the shoulder. Using the stump as a handle the blastemas were then easily removed. The blastemas, including epidermis, were washed several times in a



Fig. 1. Section through the regenerating early mound stage blastema (100X). The limits of the blastema are indicated by the dotted line just superior to the dedifferentiating humerus (H).



Fig. 2. 26 day blastema explanted onto a Millipore filter strip (MFS). Apical Cap (AC). 100X.

sterile antibiotic-antimycotic (Gibco #524) Holtfreter's solution (BSS). In studies requiring longer periods in the nerveless state, bilateral resections were performed on spinal nerves 3,4, and 5 (Singer and Sidman, 1951). This procedure effectively removed motor and sensory nerve connections to the stump while leaving the blood supply intact. To check the lack of innervation, stimulations were routinely performed on the regenerating limbs.

Culture procedures:

The blastemas, including epidermis, were explanted cut surface down onto small squares of millipore filter (type HA) supported on stainless steel grids in Falcon organ culture dishes (Falcon Plastics). The explant was oriented so that the interna, mesodermally-derived cells were exposed to the medium and to any additives therein (Fig. 3.). The medium, a modified Lebowitz L-15 incorporating the changes introduced by Stocum (1969), 0.5 ml per culture dish, contained 0.5 μ Ci/ml thymidine-methyl- 3 H, specific activity 6.7 Ci/mM (New England Nuclear) or 1.0 μ Ci/ml DL-leucine-1- 14 C, specific activity 2-10 mCi/mM (New England Nuclear). The pH was $7.35 \pm .05$ and the average osmolarity was 263 milliosmols. The culture time in all cases was 24 hours. The culture environment was a humidified atmosphere maintained at 23°C. It was considered unnecessary to change the medium during the 24 hour incubation period. Experimental cultures also contained the agent to be tested; control cultures consisted of contralateral blastemas from the same animals and were treated identically to the experimentals except for the addition of the test agent to the latter.

Dibutyryl cyclic adenosine 3', 5'-monophosphate (Calbiochem),



Fig. 3. Explanted blastema (arrow) on a Millipore filter strip (25 mm^2) in a Falcon Plastics culture dish.

when added to cultures, was used at a final concentration of 10^{-5} M. This concentration was determined to be optimal within the range of 10^{-4} to 10^{-7} M (Fig. 4). The other agents tested singly for their effect on protein and DNA synthesis were: sodium fluoride (10^{-2} M), cyclic AMP-phosphodiesterase (0.1 mg/ml; Sigma), imidazole (10^{-5} M), theophylline (10^{-3} M) and acetylcholine chloride (10^{-4} M). A total of 300 pairs of blastemas were used; control and experimental pairs were always processed simultaneously.

Tissue Measurements:

Following the 24-hour culture period the blastemas were individually rinsed, then air dried, weighed, and homogenized in 0.2 ml cold 5% trichloroacetic acid (TCA). The microhomogenizer was rinsed with additional cold TCA to yield a final volume of 1.0 ml TCA-homogenate. The solution was allowed to precipitate for 30 minutes at 0°C., then after resuspension, a 0.2 ml aliquot was spotted onto a Millipore filter disc (type HA; 0.45 μ porosity), and washed twice with 10.0 ml cold TCA under mild suction. Additional washes with hot or cold TCA or ether-ethanol do not appreciably alter the level of incorporation (Lebowitz and Singer, 1970). The discs were air dried and measured for TCA-precipitable radioactivity by liquid scintillation spectrometry. The scintillation fluid consisted of 4.0 gms Omnifluor (New England Nuclear) per liter toluene. The counting was done on a Packard Tricarb Liquid Scintillation Counter, the only correction being for background. All samples were counted at 0°C. for at least 1000 counts. The data were calculated as CPM/mg dry weight tissue, then converted to percent of control value. The difference in isotope incorporation between each member of a pair of blastemas from the same animal was treated as a single non-independent observation and analyzed

for significance, with the aid of an IBM 360 computer, by the "t" test for paired observations.

Histological Preparation:

Several cultures were examined histologically to insure the exclusion of stump tissue and as a check on the viability of the cultures. The blastema-filter assembly was rinsed several times in BSS, then fixed in Bouin's solution and stored in 70% ethanol. The tissues were embedded in Paraplast and sectioned at 10 μ . The sections were stained with Delafield's hematoxylin and eosin.

RESULTS

1. Studies on protein synthesis.

a. Relationship between dibutyryl cyclic AMP concentration and protein synthesis in early and late stage blastemas.

Regenerates reaching the mound stage (14-16 days post-amputation at 20°C.) were removed and cultured in the presence of various concentrations of dibutyryl cyclic AMP. Since preliminary studies with the naturally-occurring cyclic AMP produced no significant alteration in leucine incorporation, this study was performed so that in future investigations a concentration of nucleotide which would yield maximum response and reproducibility could be used. The concentration range investigated was 10^{-4} to 10^{-7} M. The extreme concentrations produced insignificant changes in label incorporation (Fig. 4 and 5), while a dose of 10^{-5} M provided a significant stimulation ($P < .01$; $n = 54$) of protein synthesis compared to control blastemas from the same animal. As a result of this study the concentration of dibutyryl cyclic AMP used in subsequent studies was 10^{-5} M. This concentration provided maximum stimulation of protein synthesis with no pathology noted during histological examinations.

b. The effects of dibutyryl cyclic AMP and NaF.

Incubation of mound and palette blastemas cultured in the presence of dibutyryl cyclic AMP showed a significant increase in the level of isotope incorporation (Table 1.). In the presence of the fluoride ion, which has been reported to enhance the activity of adenylate cyclase, protein synthesis was increased significantly in both the mound and palette stage and closely approximated the extent of synthetic activity observed in the presence of dibutyryl cyclic AMP (Table 1.).

Fig. 4. Dosage Study. Effects of various concentrations of dibutyryl cyclic AMP on ^{14}C -leucine incorporation by 14-16 day limb regenerates. The differences in ^{14}C -leucine incorporation were significant in blastemas treated with 10^{-5}M and 10^{-6}M concentrations ($P < .05$). The mean control value (10^{-5}M) was 131 ± 20 CPM/mg tissue. Solid line represents experimental values. Open circles represent control values. Brackets indicate S.E.M.

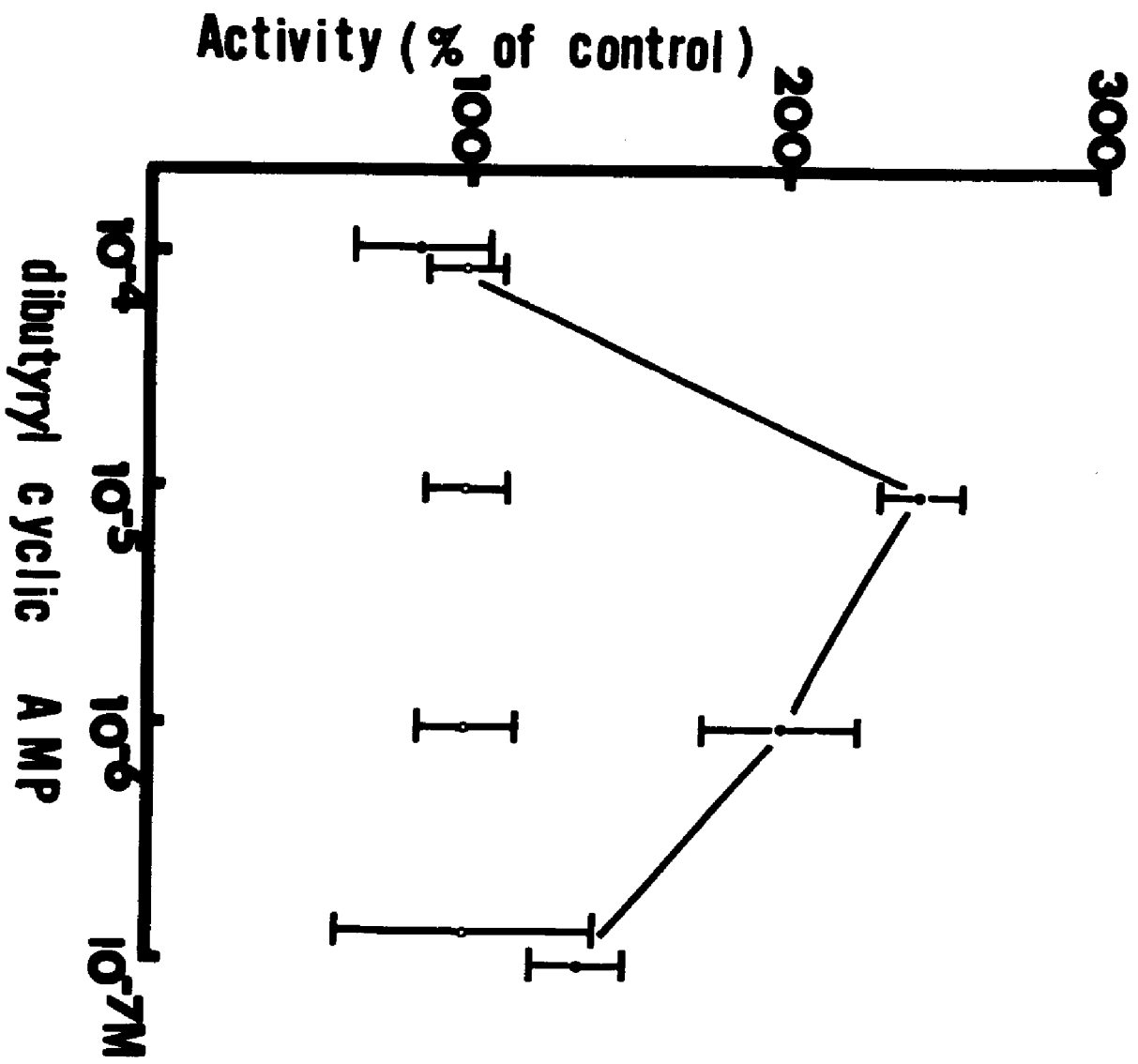


Fig. 5. Dosage Study. The effect of various concentrations of dibutyryl cyclic AMP on ^{14}C -leucine incorporation by 26-28 day limb regenerates. Mean control CPM/mg tissue (10^{-5}M) was 290 ± 35 . The difference in ^{14}C -leucine incorporation was significant in blastemas treated with 10^{-5}M dibutyryl cyclic AMP. The solid line represents experimental values. Open circles represent control values. Brackets represent the S.E.M.

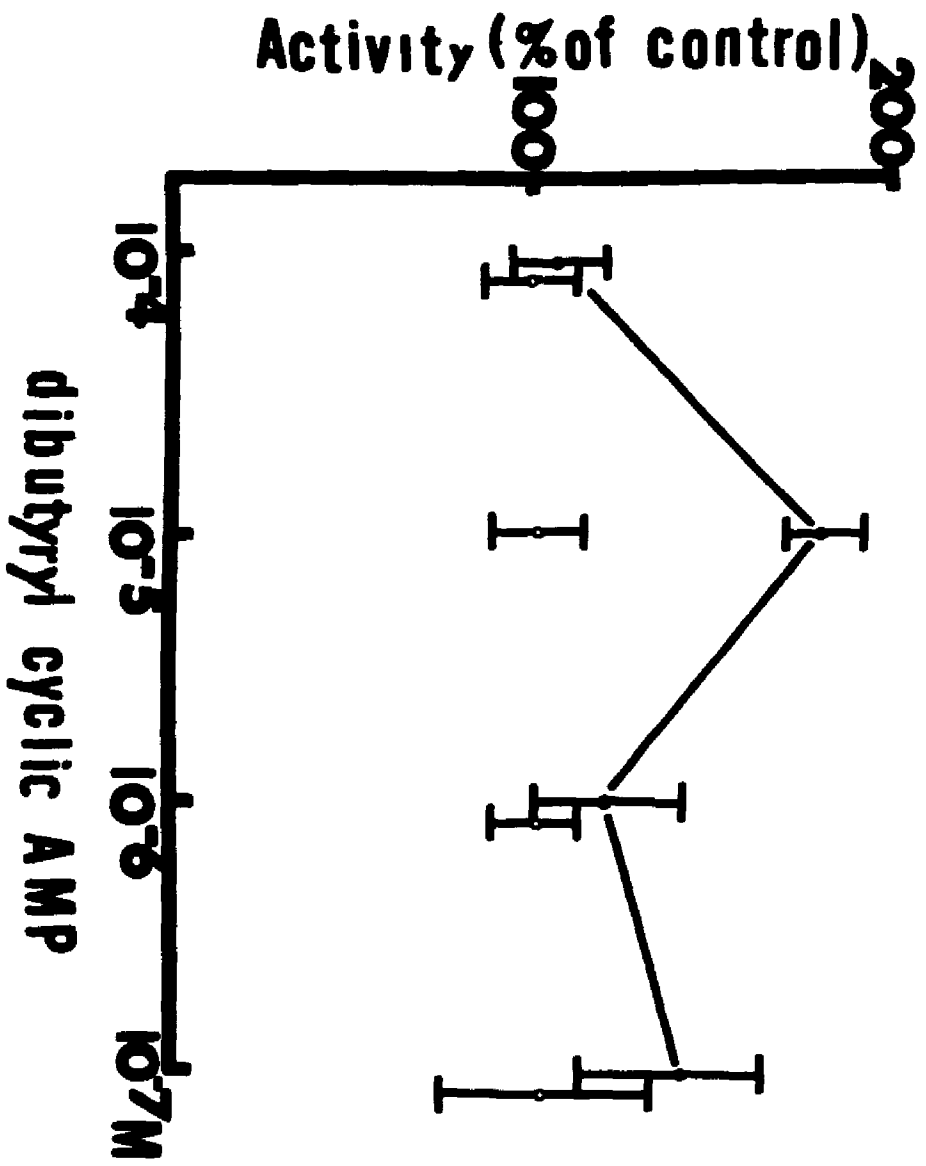


Table 1. The effects of various agents on ¹⁴C-leucine incorporation by cultured amphibian forelimb regeneration blastemas. Mean control CPM/mg tissue \pm 1 S.E.M. was 729 \pm 46 (n = 125).

Treatment	¹⁴ C-leucine incorporation (% of control value)	P value
14-16 day regenerates		
10 ⁻⁵ M dibutyryl cyclic AMP	244	<.01
10 ⁻² M NaF	181	<.01
10 ⁻³ M Theophylline	59	>.05
10 ⁻⁵ M Imidazole	88	>.05
0.10 mg/ml phosphodiesterase	122	>.05
26-28 day regenerates		
10 ⁻⁵ M dibutyryl cyclic AMP	196	<.001
10 ⁻² M NaF	173	<.01
10 ⁻³ M Theophylline	98	>.05
10 ⁻⁵ M Imidazole	76	>.05
0.10 mg/ml phosphodiesterase	112	>.05

c. The effects of agents which alter phosphodiesterase activity.

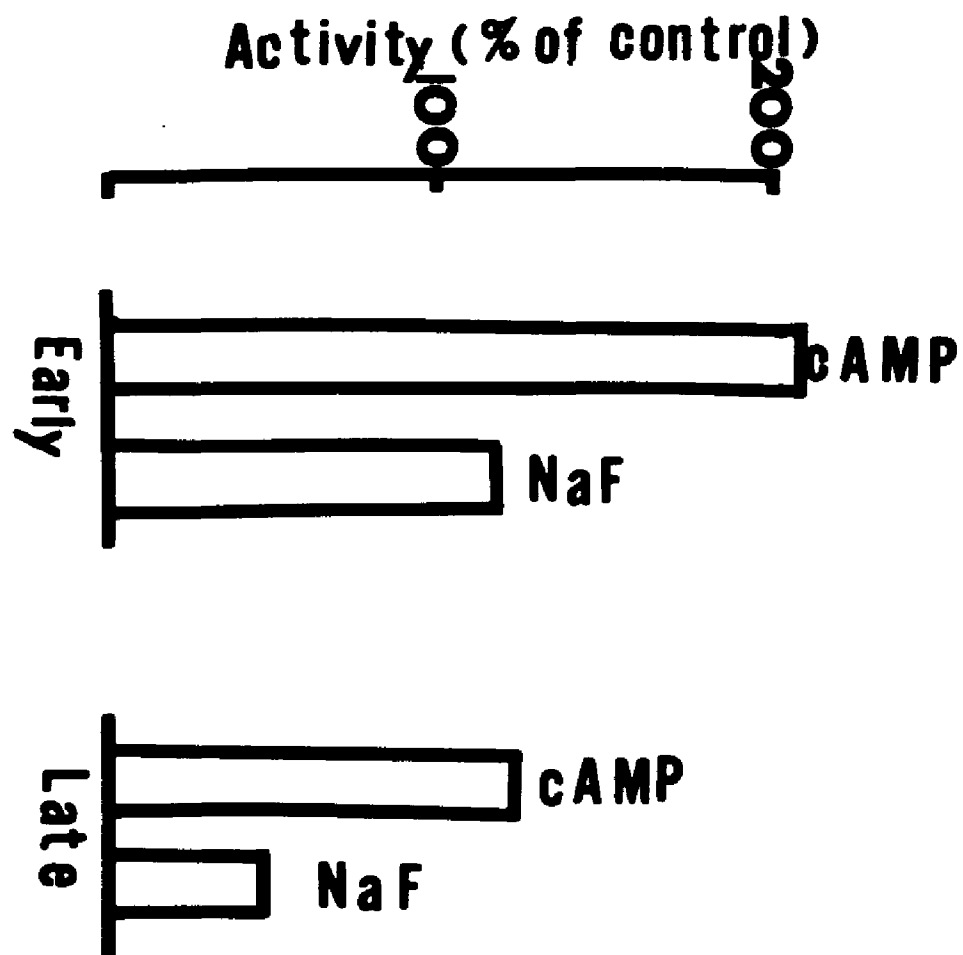
Theophylline, at a concentration of $10^{-3}M$, was used as an inhibitor of phosphodiesterase action (Butcher and Sutherland, 1962). The action of phosphodiesterase is to convert cyclic AMP into the inactive non-cyclic 5'-AMP. Swislocki (1970) reported that the dibutyryl derivative is converted very rapidly into the non-cyclic N^6 -monobutyryl form, which is homologous to the physiologically inactive form of non-cyclic AMP. Inhibition of phosphodiesterase normally raises the intracellular level of cyclic AMP and therefore potentiates the responses elicited by the nucleotide. Mound and palette blastemas cultured in the presence of theophylline showed no significant change in the level of protein synthesis. Imidazole at a concentration of $10^{-5}M$ was added to the medium to stimulate the activity of phosphodiesterase, which should reduce the level of cyclic AMP in the absence of exogenous nucleotide (Robison, Butcher, and Sutherland, 1972). In the early and late blastemas no significant change in protein synthesis due to treatment with imidazole was observed (Table 1.). Phosphodiesterase, isolated from beef heart, was tested to determine whether or not exogenous enzyme, which should decrease the level of ambient cyclic AMP, had any effect on the limb regenerate. No increase was observed with the addition of 0.1 mg phosphodiesterase/ml medium.

d. Cyclic AMP and NaF effects on denervated limbs.

The addition of exogenous dibutyryl cyclic AMP to the medium of cultured mound blastemas denervated for 72 hours before explantation significantly stimulated the total ^{14}C -leucine incorporation, while in the palette stage no apparent increase in protein synthesis was

observed (Fig. 6). NaF, which stimulates adenylate cyclase activity, failed to increase significantly the level of protein synthesis in limbs (mound stage) maintained nerveless for 72 hours, while in palette regenerates the level of protein synthesis in the presence of NaF was below the level observed in controls.

Fig. 6. Effect of dibutyryl cyclic AMP and NaF on ^{14}C -leucine incorporation by early and late regenerates denervated 72 hours prior to explantation. Mean control CPM/mg tissue was 339 (early) and 187 (late). The differences in ^{14}C -leucine incorporation were significant in early regenerates treated with dibutyryl cyclic AMP and in late regenerates treated with NaF. ($P < .05$).



2. Studies on DNA synthesis.

a. Effects of dibutyryl cyclic AMP and NaF.

From previous studies described above on the effects of dibutyryl cyclic AMP on protein synthesis, it was found that a $10^{-5}M$ solution of dibutyryl cyclic AMP provided maximum stimulation. This concentration was therefore chosen to test the effects of dibutyryl cyclic AMP on DNA synthesis. In both the mound and palette stage regenerates exposed to exogenous dibutyryl cyclic AMP there was a significant increase in 3H -thymidine incorporation (Table 2.). NaF, which potentiates the action of adenylate cyclase, produced no effects in early blastemas but did significantly enhance 3H -thymidine incorporation in the late (palette) stage blastema. This increased NaF-induced incorporation of isotope in the palette stage exceeded the level observed following treatment with exogenous dibutyryl cyclic AMP.

b. Effects of agents which alter phosphodiesterase activity.

In the mound blastema the addition of theophylline, an inhibitor of phosphodiesterase, produced a significant increase in DNA synthesis. This effect was not observed in the palette stage. The addition of the xanthine derivative, imidazole, a potentiator of phosphodiesterase, produced no observable change in DNA synthesis in either the mound or palette stage. The addition of phosphodiesterase did not affect the level of synthesis in palette regenerates but caused an increase in 3H -thymidine incorporation in early stages of regeneration (Table 2.).

c. The effects of dibutyryl cyclic AMP on denervated blastemas.

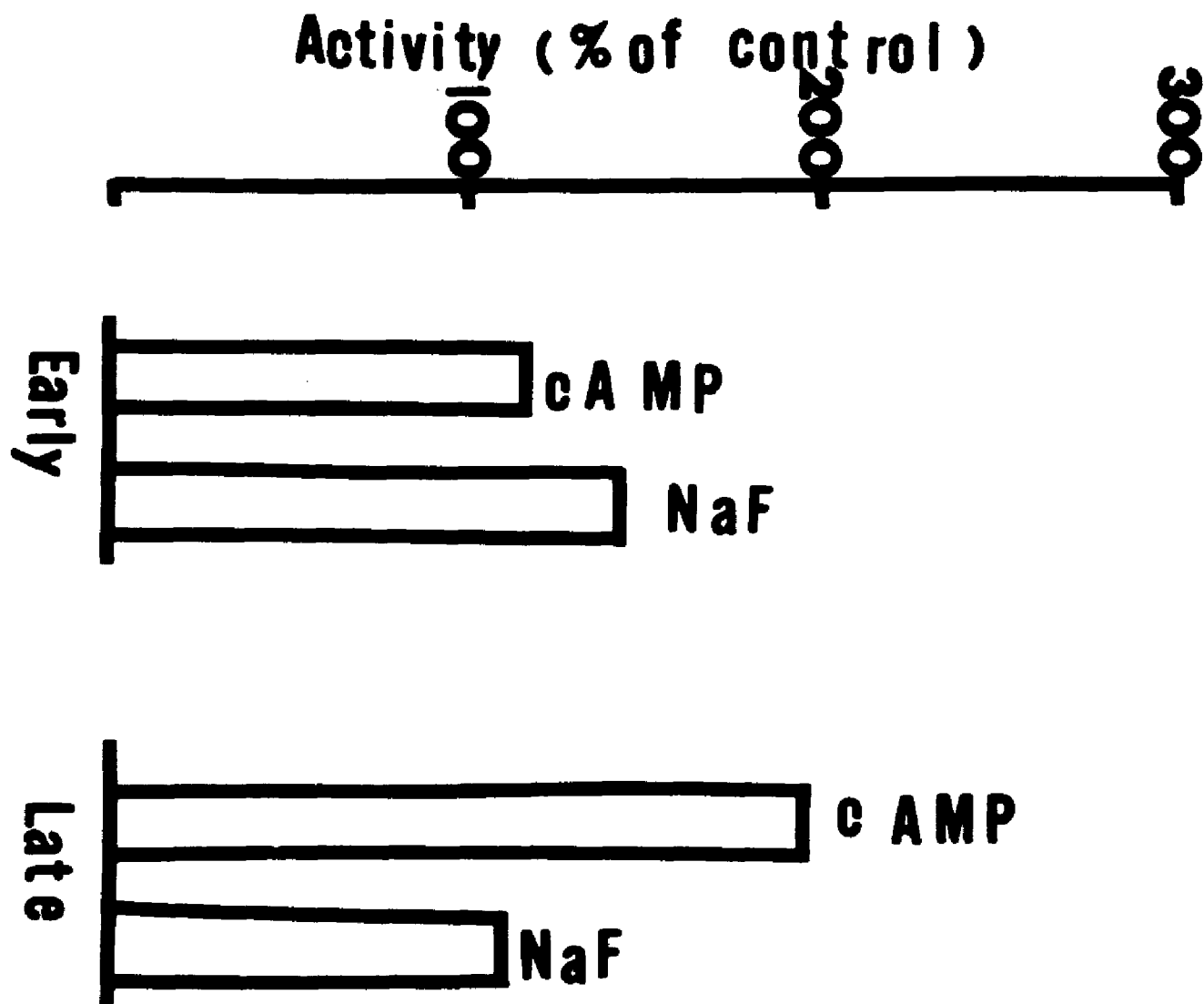
The addition of either exogenous dibutyryl cyclic AMP or NaF did not alter the total 3H -thymidine incorporation in mound

Table 2. The effects of some agents on ^3H -thymidine incorporation by cultured amphibian forelimb regeneration blastemas. Mean control CPM/mg tissue \pm 1 S.E.M. was 649 ± 129 (n = 99).

Treatment	^3H -thymidine incorporation (% of control value)	P value
14-16 day regenerates		
10^{-5}M dibutyryl cyclic AMP	125	$<.05$
10^{-3}M Theophylline	160	$<.05$
10^{-2}M NaF	119	$>.05$
10^{-5}M Imidazole	103	$>.05$
0.10 mg/ml phosphodiesterase	151	$<.05$
26-28 day regenerates		
10^{-5}M dibutyryl cyclic AMP	139	$<.05$
10^{-2}M NaF	291	$<.001$
10^{-3}M Theophylline	72	$>.05$
10^{-5}M Imidazole	94	$>.05$
0.10 mg/ml phosphodiesterase	117	$>.05$

stage blastemas previously denervated for 72 hours (Fig. 7.) However, in late blastemas (palette) denervated for the same period, a significant increase in isotope incorporation was observed in the presence of dibutyryl cyclic AMP but not with NaF (Fig. 7).

Fig. 7. Effect of dibutyryl cyclic AMP and NaF on ^3H -thymidine incorporation by early and late regenerates denervated for 72 hours prior to explantation. Mean control CPM/mg tissue was 368 (early) and 396 (late). The difference in ^3H -thymidine incorporation was significant in late regenerates treated with dibutyryl cyclic AMP ($P < .01$).



3. The effects of acetylcholine on ^3H -thymidine and ^{14}C -leucine incorporation.

The addition of acetylcholine chloride at a concentration of 10^{-4}M to the culture medium significantly depressed the level of ^{14}C -leucine incorporation in both the mound and palette stages of regeneration (Fig. 8.). The depression was more pronounced in the mound stage blastema, a time of critical neural dependency. DNA synthesis, however, was unaltered at both stages of regeneration.

Histological examination (Fig. 9.) revealed that most cells in the blastema appeared normal, except for infrequent pycnotic nuclei in the periphery of some sections. Since DNA synthesis was unaffected by the acetylcholine treatment, and since histological examination showed no substantial difference between experimental and control cultures, it was concluded that the significant decrease in protein synthesis was not due to acetylcholine toxicity.

Fig. 8. Effect of acetylcholine on ^{14}C -leucine and ^3H -thymidine incorporation by early and late limb regenerates. Mean control CPM/mg tissue was 456 ± 37 (protein), 726 ± 152 (DNA). The differences in ^{14}C -leucine incorporation were significant ($P < .01$).

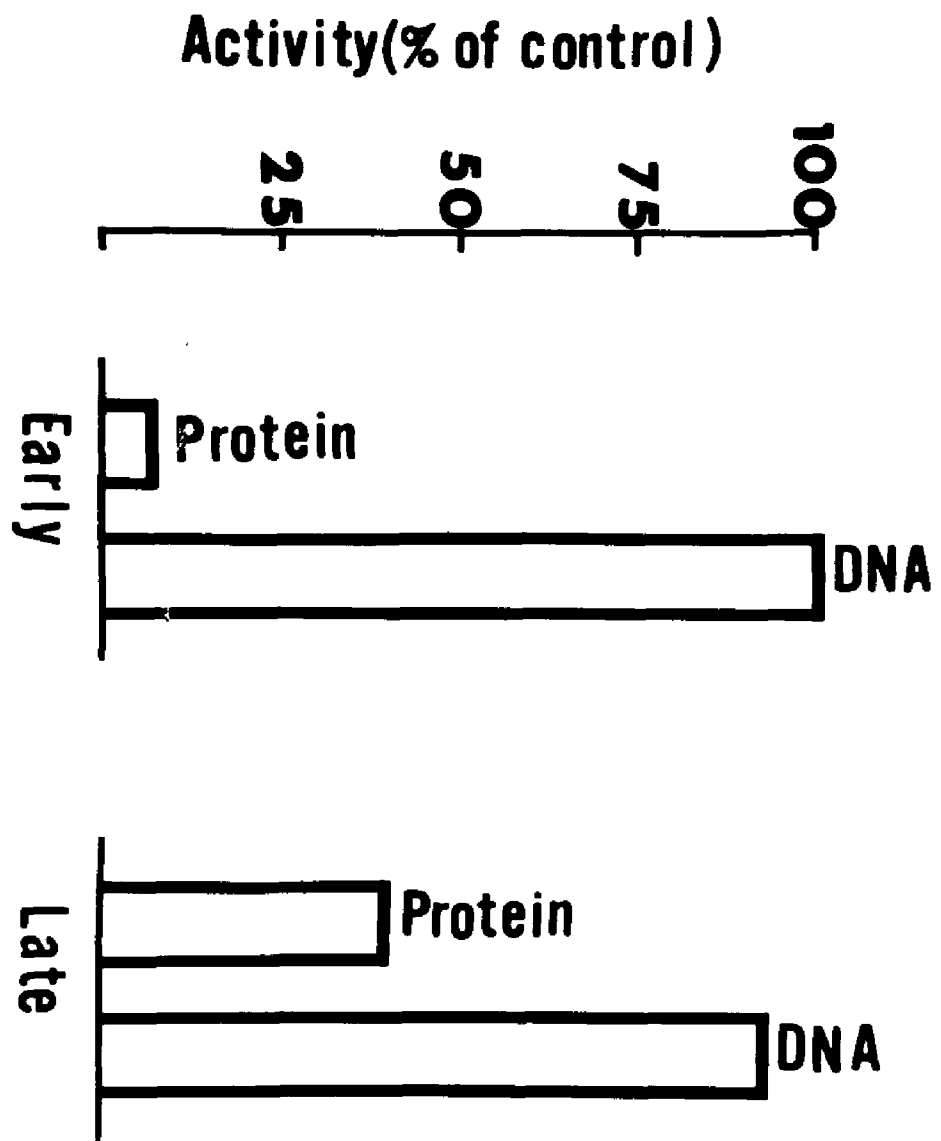




Fig. 9. 14-day blastema explanted onto a Millipore filter strip and grown in the presence of acetylcholine chloride (MFS-Millipore filter strip; arrow-mitotic figure; 900X).

DISCUSSION

I. Studies on protein synthesis.

Using the organ culture system described, forelimb blastemas can be maintained successfully for several days and will exhibit similar changes in development to those observed in vivo (Stocum, 1968). The present study demonstrates that dibutyryl cyclic AMP stimulates incorporation of ^{14}C -labelled amino acid, which indicates that protein synthetic activity within the cells has increased. This increased synthetic activity was observed in both mound and palette blastemas.

Cyclic AMP is generally reported to be relatively ineffective in intact cell preparations, a conclusion also drawn during our own initial studies. To overcome this limitation Posternak et al. (1962) have prepared analogs and derivatives of cyclic AMP to obtain substances that would readily penetrate the cell membrane or offer a greater resistance to the action of phosphodiesterases, or both. In this investigation the dibutyryl derivative was used (N^6 -2'-O-dibutyryl cyclic AMP), and was more active than the naturally occurring cyclic AMP molecule.

Blastemas grown in the presence of imidazole and phosphodiesterase, pharmacological agents that presumably decrease the level of cyclic AMP, did not show any increase in isotope incorporation into protein. Addition of NaF, which should stimulate adenylate cyclase, produced almost identical increases in protein synthesis to those observed in blastemas cultured in the presence of exogenous nucleotides. These results are compatible with those obtained by other investigators using different biological systems. Rall and Sutherland

(1958) reported that fluoride ions have been observed to stimulate adenylate cyclase in many diverse tissue preparations. However, there are systems such as in bacteria (Tao and Lipman, 1969) or developing rat brain (Schmidt, et al., 1970) in which there is no fluoride-induced stimulation of the enzyme. The underlying mechanism of fluoride stimulation is still not understood.

If regeneration is dependent upon a trophic chemical emitted from neural tissue, then there could conceivably be a time dependent loss in the level of metabolic stimulation since during the process of explantation the blastema is, in effect, totally denervated (ie. all nerve connections have been obliterated). The possibility exists that the neurotrophic factor of some related substance may still be present within the blastema at sufficient levels to elicit a short-term maintenance of protein synthesis. To test the possibility of a time-dependent loss of this postulated factor necessary for protein production, limbs were denervated 72 hours before explantation. The results demonstrate that the addition of exogenous dibutyryl cyclic AMP provides significant stimulation of protein synthetic activity at early stages. This stimulation corresponds to that observed in blastemas not denervated. In the palette stage, incorporation is increased in the presence of dibutyryl cyclic AMP, but to a lesser degree than in the mound stage. The results suggest that the addition of exogenous dibutyryl cyclic AMP supplements the time-dependent loss of the factor(s) proposed by others. Adenylate cyclase increases the level of intracellular cyclic AMP, therefore the addition of fluoride, which stimulates the enzyme, significantly increased the level of protein synthesis. After ex-

tended denervations, the fluoride ion was unable to elicit increased incorporation of ^{14}C -leucine and, in fact, caused a depression of incorporation in the later stage of regeneration, which suggests a diminished level of adenylate cyclase in the regenerate.

Exogenous phosphodiesterase was expected to lower isotope incorporation below control levels as was the addition of phosphodiesterase potentiators such as imidazole (Cheung, 1967). The results indicate that phosphodiesterase, in the concentration used, had no effect on protein synthesis within the time limits of the experiment. It is possible that the large enzyme molecule did not penetrate the blastema to an appreciable extent. It is also thought that several forms of the enzyme exist (Hardman, Butcher and Sutherland, 1971); perhaps imidazole is relatively ineffective in potentiating the urodele enzyme, if the enzyme is present.

The location of cyclic AMP action in the blastema is currently under investigation. Foret (personal communication) has obtained morphological evidence which lends support to the results discussed here. He has observed that limbs infused with theophylline, an inhibitor of phosphodiesterase, produced occasional supernumerary, ectopic blastemas. He also observed a mitogenic effect on regenerating limbs treated with dibutyryl cyclic AMP. This agrees with the evidence reported here that cyclic AMP is involved in anabolic processes which are necessary for growth in regenerating tissues.

The primary objective of this study was to analyze the effects of denervation and cyclic AMP replacement on protein synthesis in the regenerating blastema grown in vitro. Bodemer and Everett (1959) collected evidence which showed that protein synthesis is augmented

in the vicinity of the brachial nerve in the regenerating limb. The results reported here indicate that in the limb blastema an adenylate cyclase-cyclic AMP system may exist and may represent an intrinsic component of the neurotrophic factor or at least may be involved in its expression.

2. Studies on DNA synthesis.

In previous studies on limb regeneration in vivo, Foret (personal communication) obtained some evidence for a mitogenic effect of cyclic AMP and its analogue, dibutyryl cyclic AMP. Those results have been strengthened by the data obtained in the present study. One must, of course, use caution in extrapolating from an organ culture system to that which occurs in the intact animal. Nevertheless, the results observed, both in vivo and in vitro, are complementary and in agreement. A stimulation of mitosis by cyclic AMP is not surprising, for similar effects of this nucleotide on DNA synthesis and mitosis have been reported to occur in a number of other cell types. For example, bone marrow, normal thymus, and leukemic thymus cells (Burger and Knyszynski, 1971; MacManus and Whitfield, 1969; Rixon, Whitfield, and MacManus, 1970) are all stimulated into DNA synthesis or mitosis by cyclic AMP. Cyclic AMP may be involved, as well, in the regulation of growth and contact inhibition in both normal and transformed cells (Otten, Johnson, and Pastan, 1971; Sheppard, 1971). That a stimulatory effect of the nucleotide on growth is not universal, however, is suggested by work reporting an inhibition of ^3H -thymidine incorporation by normal embryonal cells (Frank, 1972) as well as neoplastic cells (Yang and Vas, 1971). Our results are somewhat paradoxical, however. Stimulation of DNA synthesis by dibutyryl cyclic AMP was apparently as easily accomplished during the late stage in regeneration (palette), a phase of rapid cell multiplication. If this period of rapid growth is regulated, at least in part, by an adenylate cyclase-cyclic AMP system, then the pronounced amplification of DNA synthesis produced by sodium fluoride is a reasonable response, for fluoride has frequently been observed to stimulate adenylate cyclase activity (Robison,

Butcher, and Sutherland, 1971). It has not, however, previously been observed to stimulate cyclic AMP formation in intact cells. Furthermore, if the adenylate cyclase system is well developed in the later stage of regeneration, one could expect theophylline, an inhibitor of cyclic phosphodiesterase, to cause an accumulation of endogenous cyclic AMP and thus an increase in ^3H -thymidine uptake. Such an effect was observed only in early stages of regeneration, however.

Finally, if adenylate cyclase were associated with nerve terminals in the regenerate, a working hypothesis now under investigation in some laboratories, it might be expected that denervation of the limb several days before excission of the blastema would result in a somewhat lower concentration of the enzyme in the region of the blastema. However, no information is available concerning the stability of adenylate cyclase in this system. If this were the case then the early regenerate should still respond to exogenous dibutyryl cyclic AMP but show no significant response to fluoride. Such an effect was in fact observed: the fluoride treated "normal" late regenerate showed nearly a 300% increase in ^3H -thymidine incorporation, compared to the control, whereas uptake by denervated, fluoride treated regenerates of the same stage was only about 115% of the control value and was not statistically significant.

Although the results reported here do not prove a role of cyclic AMP in the regulation of limb regeneration, they are suggestive of such a function. Preliminary unpublished observations by Foret (personal communication) have, in fact, demonstrated a relatively high adenylate cyclase activity in regenerating newt limbs. It is believed, therefore, that additional investigations will confirm and elucidate the mechanism by which the process of regeneration is regulated.

3. . The effects of acetylcholine on ^3H -thymidine and ^{14}C -leucine incorporation.

These results suggest that protein synthesis in the regenerating limb may be, at least in part, regulated by acetylcholine. Acetylcholine does not, however, mimic the observed effects of the nerve in vivo (Gutmann, 1970; Singer, Davis and Scheuing, 1960; Taban, 1955). Furthermore, its effect on protein synthesis is opposite to that of the high molecular weight neural factor (Lebowitz and Singer, 1970). Finally, if acetylcholine were the only regulatory factor in limb regeneration then denervation would be expected to produce an increase in protein synthesis rather than the decrease observed by Dresden (1969). There would appear, however, to be a variety of substances involved in the regulation of regenerative growth since it has recently been shown that only about 40 per cent of protein synthesis in the regenerating amphibian limb is nerve dependent (Lebowitz and Singer, 1970). Undoubtedly, then, the total synthetic activities involved in limb regeneration are mediated by a variety of humoral and hormonal factors of which acetylcholine may be but one.

BIBLIOGRAPHY

- Becker, R.O. and Spadaro, J.A. 1972. Electrical stimulation of partial limb regeneration in mammals. *Bull. N.Y. Acad. Med.* 48:627.
- Bodemer, C.W. and Everett, N.B. 1959. Localization of newly synthesized proteins in regenerating newt limbs as determined by radioactive localization of injected methionine-S³⁵. *Devel. Biol.* 1:327.
- Bryant, S.V., Fyfe, D. and Singer, M. 1971. The effects of denervation on the ultrastructure of young limb regenerates in the newt, *Triturus*. *Devel. Biol.* 24:577.
- Burger, M. and Knyszynski, A. 1971. The effect of adenosine 3', 5'-monophosphate on ³H-thymidine incorporation in vitro into mouse spleen and thymic cells. *Hoppe-Seyler's Z. Physiol. Chem.* 352:1019.
- Butler, E.G. and O'Brien, J.P. 1942. Effects of localized X-radiation on regeneration of the urodele limb. *Anat. Record* 84:407.
- Butler, E.G. and Schotte', O.G. 1949. Effects of delayed denervation on regeneration activity in limbs of urodele larvae. *J. Exp. Zool.* 112:361.
- Butcher, R.W., Ho, R.J., Meng, H.C. and Sutherland, E.W. 1965. Adenosine 3', 5'-monophosphate in biological materials. II. The measurement of adenosine 3', 5'-monophosphate in tissue and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. *J. Biol. Chem.* 240:4515.
- Butcher, R.W. and Sutherland, E.W. 1968. Effects of lipolytic and anti-lipolytic substances on adenosine 3', 5'-monophosphate levels in isolated fat cells. *J. Biol. Chem.* 243:1705.
- Butcher, R.W. and Sutherland, E.W. 1962. Adenosine 3', 5'-phosphate in biological materials. 1. Purification and properties of cyclic 3', 5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. *J. Biol. Chem.* 237:1244.
- Carlson, B. 1969. Studies on the mechanism of implant-induced supernumerary limb formation in urodeles. *J. Exp. Zool.* 164:227.
- Chalkley, D.T. 1954. A quantitative histological analysis of forelimb regeneration in *Triturus viridescens*. *J. Morphol.* 94:21.
- Cheung, W.Y. 1966. Inhibition of cyclic nucleotide phosphodiesterase by adenosine 5'-triphosphate and inorganic pyrophosphate. *Biochem. Biophys. Res. Comm.* 23:214.
- Cheung, W.Y. 1967. Properties of 3', 5'-nucleotide phosphodiesterase from rat brain. *Biochem.* 6:1079.

- Deck, J.D. and Futch, C.B. 1969. The effects of infused materials upon the regeneration of newt limbs. I. Blastemal extracts in denervated limb stumps. *Devel. Biol.* 20:332.
- DeConinck, L., Denuse, M., Diercx, Fr. and Janssens, M. 1965. Acides ribonucléique, hormone somatotrope et régénération chez Triturus (Urodèles). *Ann. Soc. Roy. Zool. Belgique.* 86:191.
- DeRobertis, E., Arnaiz, G.R.D.L., Alberici, M., Butcher, R.W. and Sutherland, E.W. 1967. Subcellular distribution of adenyl cyclase and cyclic nucleotide phosphodiesterase in rat brain cortex. *J. Biol. Chem.* 237:3487.
- Dresden, M.H. 1969. Denervation effects on newt limb regeneration: DNA, RNA, and protein synthesis. *Devel. Biol.* 19:311.
- Frank, W. 1972. Cyclic 3', 5' AMP and cell proliferation in cultures of embryonic rat cells. *Exptl. Cell Res.* 71:238.
- Gill, G.N. and Garren, L.D. 1970. A cyclic 3', 5'-adenosine monophosphate dependent protein kinase from adrenal cortex: Comparison with cyclic AMP binding protein. *Biochem. Biophys. Res. Comm.* 39:335.
- Goldberg, A.L. and Singer, J.J. 1969. Evidence for a role of cyclic AMP in neuromuscular transmission. *Proc. Nat. Acad. Sci. U.S.* 64:134.
- Greengard, P. and Costa, E. 1970. The role of cyclic AMP in cell function. New York: Raven Press.
- Gutmann, E. 1970. Open questions in the study of the "trophic" function of the nerve cell. *Top. Probl. Psychiat. Neurol.* 10:54.
- Hardman, J.G., Robison, G.A. and Sutherland, E.W. 1971. Cyclic nucleotides. *Ann. Rev. Physiol.* 33:311.
- Hay, E.D. and Fischman, D.A. 1961. Origin of the blastema in regenerating limbs of the newt Triturus viridescens. (An autoradiographic study using tritiated thymidine to follow cell proliferation and migration). *Devel. Biol.* 3:26.
- Katz, B. 1966. Nerve, muscle, and synapse. McGraw Hill. New York. pp. 114-120.
- Klein, M.I. and Makman, M.H. 1971. Adenosine 3', 5'-monophosphate-dependent protein kinase of cultured mammalian cells. *Science* 172:863.
- Konijn, T.M. 1970. Microbiological assay of cyclic 3', 5'-AMP. *Experientia* 26:367.
- Krnjevic, G. and Miledi, R. 1958. Some effects produced by adrenaline upon neuromuscular propagation in rats. *J. Physiol.* 141:291.

- Kuo, J.G. and Greengard, P. 1969. Cyclic nucleotide-dependent protein kinases. IV. Wide spread occurrences of adenosine 3', 5'-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc. Nat. Acad. Sci. U.S.* 64:1349.
- Lebowitz, P. and Singer, M. 1970. Neurotrophic control of protein synthesis in the regenerating limb of the newt, *Triturus*. *Nature* 225:824.
- MacManus, J.P. and Whitfield, J.F. 1969. Stimulation of DNA synthesis and mitotic activity of thymic lymphocytes by cyclic adenosine 3', 5'-monophosphate. *Exptl. Cell Res.* 58:188.
- Malamud, D. 1969. Adenyl cyclase: Relationship to stimulated DNA synthesis in parotid glands. *Biochem. Biophys. Res. Comm.* 35:754.
- McLennan, H. 1971. *Synaptic Transmission*. Philadelphia: Saunders (ed. 2).
- Meester, W.D. and Hardman, H.F. 1967. Blockade of the positive inotropic actions of epinephrine and theophylline by acetylcholine. *J. Pharmacol. Exp. Therap.* 158:241.
- Murad, F., Chi, Y.M., Rall, T.W. and Sutherland, E.W. 1962. Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3', 5'-phosphate by preparations from cardiac muscle and liver. *J. Biol. Chem.* 237:1233.
- Otten, J., Johnson, G.S. and Pastan, I. 1971. Cyclic AMP levels in fibroblasts: Relationship to growth rate and contact inhibition of growth. *Biochem. Biophys. Res. Comm.* 44:1192.
- Overton, J. 1950. Mitotic stimulation of amphibian epidermis by underlying grafts of CNS tissue. *J. Exp. Zool.* 115:521.
- Posternak, T., Sutherland, E.W. and Henion, W.F. 1962. Derivatives of cyclic 3', 5'-adenosine monophosphate. *Biochem. Biophys. Acta (Amst.)* 65:558.
- Rall, T.W. and Sutherland, E.W. 1958. Formation of cyclic adenine ribonucleotide by tissue particles. *J. Biol. Chem.* 232:1065.
- Rall, T.W. 1969. The role of cyclic AMP in the nervous system. In, "Neurosciences Research Progress Bulletin" 8:223.
- Rall, T.W. and Sutherland, E.W. 1962. Adenyl cyclase. II. The enzymatically catalyzed formation of adenosine 3', 5'-phosphate and inorganic pyrophosphate from adenosine triphosphate. *J. Biol. Chem.* 237:1228.
- Rasmussen, H. 1971. Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* 170:404.

- Rixon, R.H., Whitfield, J.F. and Macmanus, J.P. 1970. Stimulation of mitotic activity in rat bone marrow and thymus by exogenous adenosine 3', 5'-monophosphate (cyclic AMP). *Exptl. Cell Res.* 63:110.
- Robison, G.A., Butcher, R.W. and Sutherland, E.W. 1971. *Cyclic AMP*. New York: Academic Press.
- Robison, G.A., Butcher, R.W. and Sutherland, E.W. 1968. *Ann. Rev. Biochem.* 37:149.
- Rose, S.M. 1944. Methods of initiating limb regeneration in adult Anura. *J. Exp. Zool.* 95:49.
- Rosen, O.M. and Rosen, S.M. 1969. Properties of adenyl cyclase partially purified from frog erythrocytes. *Arch. Biochem. Biophys.* 131:449.
- Ruch, J.V. and Karcher, V. 1969. Etude comparative de l'action mitostatique du phosphate de dexaméthasone et du 3', 5' adénosine monophosphate cyclique. *C.R. Soc. Biol.* 163:803.
- Rzehak, K. and Singer, M. 1966. Limb regeneration and nerve fiber number in Rana sylvatica and Xenopus laevis. *J. Exp. Zool.* 162:15.
- Schmidt, A.J. 1968. *Cellular biology of vertebrate regeneration and repair*. Chicago: University of Chicago Press.
- Schmidt, A.J., Palmer, E.C., Dettbarn, W.D. and Robison, G.A. 1970. Cyclic AMP and adenyl cyclase in developing rat brain. *Devel. Psychobiol.* 3:53.
- Schotte', O.E. and Butler, E.G. 1941. Morphological effects of denervation and amputation in urodele larvae. *J. Exp. Zool.* 87:279.
- Schotte', O.E. and Butler, E.G. 1944. Phases of regeneration of urodele limbs and their dependence upon the nervous system. *J. Exp. Zool.* 97:95.
- Schotte', O.E. and Hall, A.B. 1952. Effects of hypophysectomy upon phases of regeneration in progress. *J. Exp. Zool.* 121:521.
- Schotte', O.E. 1961. Systemic factors in the initiation of regenerative processes in limbs of larval and adult amphibians. In "Synthesis of molecular and cellular structure". ed. D. Rudnick, pp. 161-192. New York: Ronald Press.
- Sheppard, J.R. 1971. Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3', 5'-cyclic monophosphate. *Proc. Nat. Acad. Sci. U.S.* 68:316.

- Sidman, R.L. and Singer, M. 1951. Stimulation of forelimb regeneration in the newt Triturus viridescens by a sensory nerve supply isolated from the central nervous system. Am. J. Physiol. 165:257.
- Singer, M. 1945. The nervous system and regeneration of the forelimb of adult Triturus. III. The role of motor supply, including a note on the anatomy of the brachial spinal nerve roots. J. Exp. Zool. 98:1021.
- Singer, M. 1949. The invasion of the epidermis of regenerating forelimb of the urodele Triturus by nerve fibers. J. Exp. Zool. 111:189.
- Singer, M. 1954. Apparatus for continuous infusion of microvolumes of solutions into organ and tissue. Proc. Soc. Exp. Biol. Med. 86:378.
- Singer, M. 1952. The influence of the nerve in regeneration of the amphibian extremity. Q. Rev. Biol. 27:169.
- Singer, M. 1959. The influence of nerves on regeneration. In "Regeneration in vertebrates", ed. C.S. Thornton, pp. 59-80. Chicago: Univ. of Chicago Press.
- Singer, M. and Craven, L. 1948. The growth and morphogenesis of the regenerating forelimb of the adult Triturus following denervation at various stages of development. J. Exp. Zool. 108:279.
- Singer, M., Davis, M.H. and Arkowitz, E.S. 1960. Acetylcholinesterase activity in the regenerating forelimb of adult newt Triturus. J. Embryol. Exp. Morphol. 8:98.
- Singer, M., Davis, M.H. and Scheuing, M.R. 1960. The influences of atropine and other neuropharmacological substances on regeneration of the forelimb in the adult urodele Triturus. J. Exp. Zool. 143:33.
- Singer, M. and Salpeter, M.M. 1961. Regeneration in vertebrates: the role of the wound epithelium. In "Growth in living systems", ed. M.X. Zarrow. pp. 277-311. New York: Basic Books.
- Singer, M. and Inoue, S. 1964. The nerve and epidermal apical cap in regeneration of the forelimb of adult Triturus. J. Exp. Zool. 155:105.
- Singer, M. and Green, M.R. 1966. Autoradiographic studies of uridine incorporation in peripheral nerve of the newt, Triturus. J. Morphol. 124:321.
- Solderling, T.R. and Hickenbottom, J.P. 1970. Inactivation of glycogen synthetase and activation of phosphorylase by kinase by the same cyclic 3', 5'-AMP-dependent kinase. Fed. Proc. 29:601.
- Sooy, L.E. and Mezerfreed, L. 1970. A serum macromolecule - supplemented medium for frog cell lines. Exp. Cell Res. 60:482.

- Stevely, W.S. and Stoken, L.A. 1968. Variation in phosphate content of histone in normal and irradiated tissues. *Biochem. J.* 110:187.
- Stevens, J., Ruben, L.N., Lockwood, D. and Rose, H. 1965. Implant-induced accessory limbs in urodeles: fresh, frozen, and boiled tissues. *J. Morphol.* 117:213.
- Stocum, D.L. 1968. The urodele limb regeneration blastema: A self-organizing system. 1. Differentiation in vitro. *Devel. Biol.* 18:441.
- Sutherland, E.W., Rall, T.W. and Menon, T. 1962. Adenyl cyclase. 1. Distribution, preparation, and properties. *J. Biol. Chem.* 237:1220.
- Sutherland, E.W. and Robison, G.A. 1966. The role of cyclic 3', 5'-AMP in response to catecholamines and other hormones. *Pharmacol.* 18:145.
- Taban, C. 1955. Quelques problemes de regeneration chez les urodeles. *Rev. Suisse Zool.* 62:387.
- Talwar, G.P., Segal, S.J., Gupta, S.L., Queirido, A., Kaker, S.N., Nandi, J.K., Srinivasan, C.N. and Sopari, M.L. 1965. Growth hormone, oestrogens, and RNA synthesis. *Proc. Second Int. Congr. Endocrinol.* pt. 1. pp. 14-18. New York: Excerpta Med. Found.
- Tassava, R.A. 1969. Hormonal and nutritional requirements for limb regeneration and survival of adult newts. *J. Exp. Zool.* 17:33.
- Thornton, C.S. 1938. The histogenesis of muscle in the regenerating forelimb of larval Amblystoma punctatum. *J. Morphol.* 62:17.
- Thornton, C.S. 1960. Influence of an eccentric epidermal cap on limb regeneration in Amblystoma larvae. *Devel. Biol.* 2:551.
- Thornton, C.S. 1965. Influence of the wound skin on blastemal cells aggregation. In "Regeneration in animals and related problems", eds. V. Kloritsis, and H.A.L. Trampusch. pp. 333-340. Amsterdam: North Holland.
- Thornton, C.S. 1970. Amphibian limb regeneration and its relation to nerves. *Am. Zool.* 10:113.
- Trowell, O.A. 1959. The culture of mature organs in a synthetic medium. *Exp. Cell Res.* 16:118.
- Van de Veerdonk, F.C.G. and Konijn, Th. M. 1970. The role of adenosine 3', 5'-cyclic monophosphate and catecholamines in pigment migration process in Xenopus laevis. *Acta Endocrinolog.* 64:354.

- Vaughan, M and Steinberg, D. 1963. Effect of hormones on lipolysis and esterification of free fatty acids during incubation of adipose tissue in vitro. J. Lipid Res. 4:193.
- Walsh, D.A., Perkins, J.P. and Krebs, E.G. 1968. An adenosine 3', 5'-monophosphate-dependent kinase of rabbit skeletal muscle. J. Biol. Chem. 243:3763.
- Yang, T. J. and Vas, S.I. 1971. Growth inhibitory effects of adenosine 3', 5'-monophosphate on mouse leukemia L-5178-Y-R cells in culture. Experientia 27:442.

APPENDIX

Experimental data - ^{14}C -leucine incorporationCPM \pm 1 S.E.M./mg tissue

Treatment	Expt'l	Control	% Control Activity	P value
-----------	--------	---------	--------------------	---------

14-16 day regenerates

Dibutyryl cyclic AMP	319 \pm 34	131 \pm 20	244	.006
Imidazole	267 \pm 45	303 \pm 32	88	.10
Phosphodiesterase	433 \pm 51	356 \pm 55	122	.26
Theophylline	321 \pm 52	548 \pm 111	59	.07
NaF	461 \pm 38	255 \pm 30	181	.007

26-28 day regenerates

Dibutyryl cyclic AMP (run 1)	282 \pm 45	128 \pm 17	220	.02*
Dibutyryl cyclic AMP (run 2)	519 \pm 54	290 \pm 35	179	.003*
Imidazole	204 \pm 51	269 \pm 38	76	.06
Phosphodiesterase	185 \pm 19	166 \pm 25	112	.60
Theophylline	224 \pm 31	229 \pm 27	98	.90
NaF	414 \pm 69	239 \pm 36	173	.004

14-16 day denervated regenerates

Dibutyryl cyclic AMP	277 \pm 37	131 \pm 15	211	.01
NaF	641 \pm 69	547 \pm 31	117	.29

26-28 day denervated regenerates

Dibutyryl cyclic AMP	208 \pm 34	177 \pm 32	118	.59
NaF	99 \pm 8	196 \pm 12	50	.004

*

P (combined) = .001

Experimental data - ^3H -thymidine incorporationCPM \pm 1 S.E.M./mg tissue

Treatment	Expt'l	Control	% Control Activity	P. value
-----------	--------	---------	--------------------	----------

14-16 day regenerates

Dibutyryl cyclic AMP	332 \pm 71	265 \pm 48	125	.05
Imidazole (run 1)	233 \pm 18	476 \pm 129	49	.19
Imidazole (run 2)	2342 \pm 362	1804 \pm 276	130	.18
Phosphodiesterase	819 \pm 120	543 \pm 88	151	.03
Theophylline	608 \pm 106	380 \pm 83	160	.02
NaF	690 \pm 175	579 \pm 181	119	.60

26-28 day regenerates

Dibutyryl cyclic AMP	233 \pm 38	168 \pm 27	139	.05
Imidazole	824 \pm 62	881 \pm 325	94	.87
Phosphodiesterase	161 \pm 25	138 \pm 16	117	.57
Theophylline (run 1)	1853 \pm 455	2554 \pm 335	73	.30*
Theophylline (run 2)	309 \pm 80	428 \pm 83	72	.13*
NaF	1169 \pm 115	402 \pm 63	291	.001

14-16 day denervated regenerates

Dibutyryl cyclic AMP	323 \pm 59	274 \pm 40	118	.13
NaF	668 \pm 89	463 \pm 60	144	.06

26-28 day denervated regenerates

Dibutyryl cyclic AMP	466 \pm 81	236 \pm 64	197	.005
NaF	643 \pm 153	556 \pm 129	116	.22

*

P (combined) = .16