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THE REGULATION OF ADENOSINE 3',5'-MONOPHOSPHATE CONTENT IN EHRLICH CELLS

BY

GILLES J. LAUZON

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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EDMONTON, ALBERTA

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE REGULATION OF ADENOSINE 3',5'-MONOPHOSPHATE CONTENT IN EHRLICH CELLS, submitted by Gilles J. Lauzon in partial fulfilment of the requirements for the degree of Master of Science.

Supervisor

Date 22 May, 1975

TO MY MOTHER AND FATHER

ABSTRACT

The manipulation of intracellular cAMP content in malignant cells is of special interest in view of the current hypothesis that a relationship exists between this cyclic nucleotide and parameters of cellular growth and differentiation. The present work was thus concerned with the elucidation of the mechanisms which regulate the cAMP content of Ehrlich ascites tumour cells incubated *in vitro* or grown intraperitoneally in mice after epinephrine or prostaglandin E_1. In particular, the time course of the elevation of cAMP in Ehrlich cells incubated *in vitro* was followed after the addition of these hormones.

The cAMP content was significantly elevated in Ehrlich cells within 10 sec after the addition of epinephrine (10^{-6} M). Peak cAMP levels were reached at about 1 min and a pronounced subsequent decline of cAMP was then observed despite the continued presence of hormone. A similar time course was observed after addition of prostaglandin E_1. This pattern of cAMP elevation and decline after hormonal stimulation has been reported in other cell or tissue slice systems as well. The major goal of the present work was to provide an explanation for the abrupt secondary decline of cAMP in Ehrlich cells despite the continued presence of hormone.

The time course of the elevation of cAMP content in Ehrlich cells after addition of epinephrine was studied *in vitro* in the presence of theophylline (1 mM); the phosphodiesterase inhibitor greatly enhanced the response to epinephrine throughout the entire time course. However, qualitative aspects of the response were the same in the presence and absence of theophylline, suggesting that the

mechanism of the secondary decline did not involve the regulation of cAMP degradation by a theophylline-sensitive phosphodiesterase.

Leakage of cAMP into the extracellular medium could not be demonstrated within periods up to 120 min after addition of epinephrine. The observed time course was not peculiar to the standard incubation medium employed since the response to epinephrine of Ehrlich cells suspended in freshly prepared ascites fluid was virtually identical.

Further addition of epinephrine during the secondary phase of decline of cAMP content in epinephrine-stimulated cells (10^{-6} M, 10 min) had little effect on the declining levels of cAMP. Hence, extracellular breakdown of hormone cannot account for the observed decline of cAMP. Furthermore, incubation medium from hormone-treated cell suspensions retained the ability to elevate cAMP content maximally when added to freshly isolated Ehrlich cells. Therefore, no inhibitory activity was associated with the extracellular medium and the hormone was still present and active.

Propranolol (10^{-5} M) added to cellular suspensions incubated for 10 min in the presence of 10^{-6} M epinephrine caused cAMP content to fall more rapidly. Propranolol alone at the same concentration did not affect resting cAMP content. Therefore, adenylate cyclase was still epinephrine-stimulated during the time when cAMP levels were falling. This is also evident from the fact that cAMP rose sharply upon addition of theophylline to cells preincubated with epinephrine. The addition of theophylline (1 mM) at various times to cells incubating in the presence of epinephrine (10^{-6} M) indicated that the magnitude of the abrupt rise of cAMP continually

decreased with time. It was concluded that either cAMP degradation became increasingly dependent on a phosphodiesterase resistant to inhibition by theophylline or the level of adenylate cyclase activity in the presence of hormone decreased with time.

Ehrlich cells incubated in the presence of 10^{-6} M epinephrine for 10 to 60 min and subsequently washed and resuspended in fresh medium were partially refractory to the effects of a second treatment with the same hormone. This phenomenon, namely a partial refractoriness to epinephrine was time-dependent and appeared to be initiated when cAMP was raised at least three fold above basal levels.

Adenylate cyclase prepared from cells incubated *in vitro* for 10 min in the presence of 10^{-6} M and 10^{-5} M epinephrine displayed approximately 50% and 60% inhibition of epinephrine-stimulated activity, respectively, compared to cyclase from cells incubated in the absence of epinephrine. Basal and NaF-stimulated activities were essentially unaffected as a result of pretreating cells with epinephrine. It is suggested that a hormone or cAMP-induced alteration of the adenylate cyclase system itself results in a lack of sensitivity to the effects of epinephrine, and that this alteration is the cause of the early phase of decline of cAMP levels in intact cells. A model is proposed which suggests that a cAMP-dependent, perhaps membrane-bound, protein kinase catalyses the phosphorylation of a component of adenylate cyclase, thus rendering it refractory to hormonal stimulation.

Epinephrine alone or in combination with theophylline similarly elevated cAMP content in Ehrlich cells growing intraperitoneally in mice. The early events in the time course of cAMP elevation

following epinephrine *in vivo* were similar to those observed *in vitro*. However, the effects of the drugs on cAMP content in Ehrlich cells *in vivo* were of shorter duration than those observed *in vitro*. It is likely that redistribution and metabolism of the drugs by the animals were responsible for this difference.

TABLE OF CONTENTS

Page No.

1. INTRODUCTION 1

1.1 General

1.2 The control of cAMP levels in cells

1.2.1 Adenylate cyclase

1.2.2 Cyclic nucleotide phosphodiesterase

1.2.3 Compartmentalization of adenine nucleotides

1.2.4 cAMP-dependent protein kinases

1.3 Some tissue or cell responses to hormonal treatment

2. METHODS 12

2.1 Tritiated cAMP solution

2.2 Propagation of Ehrlich ascites cells

2.3 Removal and *in vitro* incubations of Ehrlich cells

2.4 Acid extraction of Ehrlich cells

2.5 Purification of cAMP in cellular extracts

2.6 cAMP assays

2.7 *In vivo* experiments

2.8 Adenylate cyclase assays

3. RESULTS AND DISCUSSION 32

3.1 Time course of elevation of cAMP following epinephrine *in vitro*

3.2 Absence of extracellular accumulation of cAMP

3.3 Pharmacological properties of the catecholamine receptor in Ehrlich cells

Page No.

3.4 Time course of elevation of cAMP following prostaglandin E_1 *in vitro*

3.5 Dose-response behaviour to epinephrine and prostaglandin E_1

3.6 Effect of a second addition of hormone

3.7 Search for inhibitory activity possibly associated with incubation medium

3.8 Effect of propranolol added after epinephrine

3.9 Effect of theophylline added after epinephrine

3.10 Partial refractoriness of stimulated cells to the effects of epinephrine

3.11 Hormone-dependent modification of adenylate cyclase

3.12 A proposed mechanism for hormone-induced desensitization of adenylate cyclase

3.13 Hormonal stimulation of Ehrlich cells *in vivo*

3.14 Time course of the cAMP response of Ehrlich cells to epinephrine *in vivo*

4. GENERAL DISCUSSION 72

5. FUTURE PROJECTIONS 77

REFERENCES 80

APPENDIX A 86

LIST OF TABLES

TABLE Page

1. The effect of the method of killing and tumour extraction on cAMP content in Ehrlich cells 29
2. Effect of epinephrine-pretreatment of isolated Ehrlich cells on adenylate cyclase 62
3. The effect of epinephrine and theophylline on cAMP levels in Ehrlich cells *in vivo* 68
4. Time course of cAMP response of Ehrlich cells *in vivo* to epinephrine and theophylline 70
5. Effect of cAMP and theophylline treatments on the size of Ehrlich ascites tumours 87
6. Effect of epinephrine and theophylline on the size of Ehrlich ascites tumours 90

LIST OF FIGURES

FIGURE		Page
1.	Purity of radiolabelled cAMP	14
2.	Elution of ^{3}H-cAMP and ATP from AG 50W-X4 cation exchange resin	19
3.	DEAE-Purification of cardiac binding protein	22
4.	cAMP assay calibration curve	26
5.	Time course of the cAMP response to 10^{-6}M epinephrine	35
6.	Time course of cAMP response to 10^{-5} and 10^{-7}M epinephrine	37
7.	Time course of cAMP response to epinephrine of Ehrlich cells suspended in ascites fluid	39
8A	Effect of propranolol on the cAMP response to epinephrine	42
8B	Effect of phentolamine on the cAMP response to epinephrine	42
8C	Time course of cAMP response to orciprenaline	42
9.	Time course of cAMP response to prostaglandin E_1	43
10A	Dose-response behaviour of cAMP response to epinephrine	45
10B	Dose-response behaviour of cAMP response to prostaglandin E_1	45
11.	Effect of a second addition of epinephrine	47

FIGURE | Page

12. (A and B) Ability of medium from a hormone-treated cell suspension to elevate cAMP in fresh Ehrlich cells 49

13. Fate of adenosine added to Ehrlich cell suspensions 50

14. Effect of a late addition of propranolol 52

15. cAMP response of epinephrine-treated cells to late additions of theophylline 54

16. Ability of epinephrine-treated Ehrlich cells to respond to further additions of hormone 56

17. Dose-dependence of desensitization of Ehrlich cells to hormonal action 58

18. Response of adenylate cyclase prepared from untreated and epinephrine-pretreated Ehrlich cells to stimulation by epinephrine 64

19. Group distribution for the packed tumour cell volume per mouse for each group from Table 6 92

LIST OF ABBREVIATIONS

AMP: adenosine 5'-monophosphate

ATP: adenosine 5'-triphosphate

^{14}C: carbon-14

^{0}C: degree Centigrade

cAMP: adenosine 3',5'-cyclic monophosphate

Ci: curie

cm: centimeter

cpm: counts per minute

d: dextro

DEAE: diethylaminoethyl cellulose

EDTA: ethylenediamine-tetraacetic acid

EGTA: ethylene glycol-bis(β-amino-ethyl ether)N,N'-tetraacetic acid

g: gram

g: gravitational force

^{3}H: tritium (tritiated)

kg: kilogram

l: litre

l: levo

M: molar

meq: milliequivalent

mg: milligram

min: minute

ml: milliliter

mM: millimolar

mmole: millimole

N: normal

nm: nanometer

OD: optical density

^{32}P: phosphate-32

pg: picogram

pmole: picomole

sec: second

Tris: 2-amino-2-(hydroxymethyl)-1,3-propandiol

μCi: microcurie

μg: microgram

μl: microliter

μmole: micromole

v/v: volume to volume ratio

w/v: weight to volume ratio

1. INTRODUCTION

1.1 General

1.2 The control of cAMP levels in cells

1.2.1 Adenylate cyclase

1.2.2 Cyclic nucleotide phosphodiesterase

1.2.3 Compartmentalization of adenine nucleotides

1.2.4 cAMP-dependent protein kinases

1.3 Some tissue or cell responses to hormonal treatment

1. INTRODUCTION

1.1 *General*

Adenosine-3',5'-cyclic monophosphate (cAMP) has been shown to inhibit the growth of many neoplastic mammalian cell lines in culture (Ryan and Heidrick, 1968; Heidrick and Ryan, 1970; Heidrick and Ryan, 1971; Otten *et al.*, 1971; Johnson *et al.*, 1972; Prasad and Sheppard, 1972; Smets, 1972; Wijk *et al.*, 1972; Teel and Hall, 1973; Nagyvary *et al.*, 1973). Present evidence from studies performed in tissue culture suggests that cAMP may play a regulatory role in the complex mechanisms which control cellular growth. The presence of cAMP or agents which increase intracellular cAMP in the culture medium of malignant cells causes an apparent phenotypic reversion to normal characteristics as evidenced by a restoration of normal morphology and contact inhibition of growth and movement. Furthermore, malignant cells treated in culture for periods of days with cAMP or its derivatives display much suppressed tumourigenicity when implanted into acceptable hosts (Reddi and Constantinides, 1972; Smith and Handler, 1973). However, the effect of increased cAMP content in malignant cells *in vivo* is poorly defined. Studies exploring the carcinostatic action of cAMP *in vivo* are few in number (Gericke and Chandra, 1969; Keller, 1972; Seller and Benson, 1973) and are complicated by a possible regulatory function of cAMP at the level of the immunological response of a host to an implanted tumour (MacManus and Whitfield, 1969; Leahy *et al.*, 1970; Rigby, 1972). Furthermore, treatment schedules to date have been assigned randomly with little or no knowledge of the effects of such treatment on intracellular cAMP

levels or other parameters.

In view of our laboratory's interest in the possibility that the rate of growth of tumours *in vivo* can be affected by manipulation of their cAMP content, it became imperative to first investigate the factors which regulate the intracellular levels of the cyclic nucleotide. The selective use of hormones offers a direct, physiological means to elevate cAMP levels in a given normal or transformed cell type. As a model system, we have chosen the Ehrlich ascites tumour cell. This cell line can be routinely grown intraperitoneally in mice; thus, the tumour cells can be subjected to selective drug treatments with a minimum of interference by the host.

The adenylate cyclase system from Ehrlich cells has been previously studied and characterized in this laboratory; epinephrine and prostaglandin E_1 were found to stimulate its activity (Bär and Henderson, 1972). Accordingly, at the onset of the present thesis, we planned to study the dose relationships and time functions of the effects of these hormones on cAMP levels in intact Ehrlich cells in the hope of devising a rational approach to the manipulation of intracellular cAMP content and secondly of tumour growth parameters *in vivo*.

In the course of initial studies we became aware of a very intriguing phenomenon. Following the addition of epinephrine to Ehrlich cells incubated *in vitro*, cAMP content was very significantly elevated within 10 sec and reached a peak level at about 1 min. Thereafter, an abrupt decline of cAMP content was initiated despite the continued presence of hormone. In view of the importance of this very early decline of cAMP in the proposed studies on the carcinostatic

function of the cyclic nucleotide, our efforts were thereafter diverted toward the elucidation of the mechanisms involved in the establishment of the secondary decline of cAMP in Ehrlich cells. This study constitutes the core of the present thesis. Additional but incomplete investigations on the growth of Ehrlich cells in mice are included as an appendix.

1.2 *The control of cAMP levels in cells*

The cAMP content within a biological system can be manipulated at the level of its synthesis from ATP catalysed by the adenylate cyclase system or at the level of its breakdown to AMP catalysed by cyclic nucleotide phosphodiesterase. This section will also discuss evidence supporting subcellular compartmentalization of adenine nucleotides and its significance. Finally, the role of cAMP-dependent protein kinases will be discussed briefly.

1.2.1 *Adenylate cyclase*

The existence of adenylate cyclase has been demonstrated in a very large number of cells and tissues including many lower forms. The enzyme is associated with the cell membrane and the active catalytic site is located on the inside of the membrane (Trams and Lauter, 1974).

Although the rate of synthesis of cAMP by a given cell or tissue can be changed by a large number of external factors, hormonal influence has certainly been the major focus of attention. It is clear that the receptors with which many hormones combine to effect a response are in close association to, or an integral part of, the

adenylate cyclase system. The result of such hormone-receptor interactions are a decrease or increase of intracellular cAMP levels, depending on whether adenylate cyclase is inhibited or stimulated. The specificity of agents which influence adenylate cyclase prepared from a given tissue provides the basis for the selective physiological and pharmacological effects of many hormones and drugs. The protein components of adenylate cyclase which perform regulatory and catalytic functions are presumably associated in a complex, incompletely understood fashion within the structure of the cell membrane.

1.2.2 *Cyclic nucleotide phosphodiesterase*

Cyclic nucleotide phosphodiesterase has been demonstrated in a large number of tissues (Butcher and Sutherland, 1962). However, detailed studies of this enzyme have been performed only recently. It has now been demonstrated that at least two different forms of cAMP-specific phosphodiesterases exist with low (1 µM) and high (0.1 mM) Km values in crude brain fractions (Brooker *et al.*, 1968) and fat cells (Murad *et al.*, 1970). The low Km activity may be of major importance in the regulation of basal and stimulated cAMP content *in vivo*. Similarly, multiple forms of phosphodiesterase activity separable by electrophoresis, gel filtration or distinguishable by differing kinetic parameters have been demonstrated in many other tissues (Ryan and Heidrick, 1974). Furthermore, phosphodiesterase activity in brain homogenates has been found in both particulate and soluble fractions, the low Km enzyme being in the soluble fraction (De Robertis *et al.*, 1967).

The cAMP content of a biological system can be manipulated via inhibition or stimulation of phosphodiesterase. The methylxanthi-

nes, namely theophylline and caffeine, are potent inhibitors of phosphodiesterase (Butcher and Sutherland, 1962); imidazole, on the other hand, stimulates the enzyme (Cheung, 1967). Unfortunately, these agents are not specific and may have other actions possibly unrelated to the cAMP system.

1.2.3 *Compartmentalization of adenine nucleotides*

Relatively recent studies have suggested that adenine nucleotide compartmentalization may be yet another factor involved in the regulation of cAMP synthesis and subcellular distribution. Experiments subjecting tissues or cells to labelled adenine nucleotide precursors have indicated that cAMP is derived from specific nucleotide pools which exhibit turnover rates different from those of bulk cellular ATP. In particular, Shimizu *et al.* (1970) have shown that in guinea pig cerebral cortical slices cAMP is derived from a precursor pool of adenine nucleotide which is labelled with ^{14}C-adenine more rapidly than the bulk cellular ATP. Incubation with ^{14}C-adenosine labelled these cAMP-specific precursor pools less selectively (Shimizu *et al.*, 1970a). Other evidence was obtained from rat leg muscle cell cultures which were double labelled by the addition of ^{14}C-adenine and ^{32}P-inorganic phosphate to the culture medium (Reporter, 1972). Whereas the specific activity of the isolated ^{14}C-cAMP was similar to that of the bulk cellular ^{14}C-ATP at equilibrium, the specific activity of ^{32}P from cAMP was 10 fold greater than that from bulk cellular ATP after 15 min. The author suggests that a specific ATP pool with rapid turnover within the cell membrane provides the necessary substrate for cAMP synthesis. That model is further supported by the fact that the specific activity of ^{32}P in cAMP was increa-

sed threefold after modification of the plasma membrane by lysolecithin. The latter treatment did not affect the specific activity of total cellular ^{32}P-ATP.

It is conceivable that these specific ATP pools which serve as substrate for adenylate cyclase could provide yet another level of regulation of intracellular cAMP content. No detailed information is presently available on the amount of ATP immediately available for cAMP synthesis. Are these ATP pools specific for cAMP synthesis depleted readily? It is known that concentrations of adenine nucleotides decrease markedly in rat glial cells following repeated stimulation with norepinephrine(Schultz *et al.*, 1972). Can depletion of these specific ATP pools play a physiological role via alteration of cAMP content? It is possible that some extracellular influences affect the amount of ATP available for cAMP synthesis and thus the total cellular cAMP.

In view of the suggested existence of adenine nucleotide pools specific for cAMP formation, we can expand to a concept involving specific cAMP pools. Actual physical subcellular barriers to confine the cyclic nucleotide need not exist for compartmentalization since specific binding to macromolecular species, proteins in particular, could perform this function. It is known that the Km for the association of cAMP to the regulatory unit of protein kinase is approximately 10^{-8}M (Walton and Garren, 1970). Hence, much of the total cellular cAMP could be associated with binding protein. A heterogeneous subcellular distribution of protein kinase could thus effect the compartmentalization of cAMP itself. In this associated form, cAMP would be protected from breakdown by phosphodiesterase.

Begging for further experimentation, this area provides many interesting starting points for projects intended to explore the existence of specific adenine nucleotide pools and their significance in biochemical regulation.

1.2.4 *cAMP-dependent protein kinases*

The recent discovery of protein kinases stimulated by cAMP in various tissues has suggested that the effects of cAMP in cells might result from protein kinase activity. Many kinases which catalyse the phosphorylation of casein, protamine or histone by ATP are activated by physiological concentrations of cAMP (Kuo and Greengard, 1969 and 1970). It is clear at present that protein kinases which are activated by cAMP are composed of regulatory and catalytic subunits. The regulatory subunit tends to inhibit the activity of the catalytic subunit. By a physical association with the regulatory subunit, cAMP effects the dissociation of the catalytic and regulatory subunits, thus removing the inhibitory force on the former (Gill and Garren, 1970; Reimann *et al.*, 1971). The ability of the regulatory subunit to selectively bind cAMP has been employed by Gilman (1970) as the basis for an assay for cAMP. That method was employed in the present study and will be described in detail under Methods.

1.3 *Some tissue or cell responses to hormonal treatment*

Common observations from the time functions of hormone-dependent increases of cAMP levels in intact tissues *in vitro* are a quick rise of cAMP content toward a short lived maximum, followed by a secondary decline of variable duration toward basal levels despite the continued presence of hormone. Such time functions have been

described for example in fat cells after epinephrine (Manganiello *et al.*, 1971), in perfused rat heart after epinephrine (Robison *et al.*, 1965), in slices of rabbit cerebellum (Kakiuchi and Rall, 1968) and in diaphragm muscle after epinephrine (Craig *et al.*, 1969), in guinea pig cerebral cortical slices after histamine treatment (Schultz and Daly, 1973) and in liver slices after epinephrine (Sutherland *et al.*, 1965). However, to the author's knowledge, the mechanism of the secondary decline has been elucidated conclusively in only one system while work on two other cell types provide possible mechanisms for this phenomenon. These are discussed below.

Manganiello *et al.* (1971) have demonstrated that the effect of epinephrine on cAMP content in fat cells is transient. Following the addition of hormone, cAMP content is elevated and reaches a peak at approximately 4 min. However, by 10 min, cAMP content has declined to near resting levels despite the continued presence of hormone. The addition of propranolol to fat cells during the phase of decline caused cAMP content to fall more rapidly than in the presence of hormone alone. Since propranolol alone had no effect on cAMP levels, the authors suggest that fat cell adenylate cyclase was still epinephrine-stimulated during the phase of decline. Furthermore, a second addition of epinephrine during the phase of decline neither elevated nor prevented the further decline of cAMP levels. Therefore, breakdown of hormone does not account for the intriguing fall of cAMP content.

Further experiments revealed that stimulated fat cells which were washed and resuspended in fresh medium gained normal sensitivity to hormone. These experiments and those of Ho and Su-

therland (1971) suggested the association of a hormone antagonist with the incubation medium of fat cells. The latter reported the inhibitor released was equivalent to nonamolar concentrations of prostaglandin E_1 but, unlike this hormone, did not increase cAMP levels in spleen slices. More recently, Schwabe *et al.* (1973) have conclusively demonstrated the hormone antagonist released into the incubation medium of fat cells to be adenosine. It is not clear whether the release and hormone antagonism of this nucleoside is of any physiological significance or simply an artifact of the experimental situation *in vitro*.

In contrast to the fat cell system, human diploid fibroblasts pretreated with isoprenaline or prostaglandin E_1, hormones which increase cAMP content in this system, display a striking desensitization to the effects of the same hormone on cAMP levels when rinsed and resuspended in fresh medium (Franklin and Foster, 1973). Interestingly, the desensitization induced by these hormones is specific; that is, cells pretreated with isoprenaline continue to respond normally to prostaglandin E_1 and *vice versa*.

Accordingly, adenylate cyclase prepared from peritoneal macrophages previously treated in culture with epinephrine or prostaglandin E_2 exhibited a greatly reduced response to the same hormone used in the pretreatment (Remold-O'Donnell, 1974). Hence, preincubation of macrophages with epinephrine greatly reduced the subsequent response of broken cell adenylate cyclase to epinephrine but basal, NaF- and prostaglandin E_2-stimulated activities remained unchanged. Similarly, pretreatment of macrophages with prostaglandin E_2

specifically reduced the subsequent response of adenylate cyclase to prostaglandin E_2 but not to epinephrine. Hence, a hormone-induced desensitization of adenylate cyclase has been shown in this system. Such a mechanism could materialize many of the yet unexplained secondary declines of cAMP levels in tissues or cells subjected to hormones which stimulate the respective adenylate cyclases.

Similarly, a hormone-dependent desensitization of adenylate cyclase to hormone is described in Ehrlich cells in the present work. However, the author was not aware of the similar phenomenon shown previously in macrophages (Remold-O'Donnell, 1974) when he undertook experiments to explore a possible effect of hormonal treatment directly on the adenylate cyclase system. However, the positive results that we obtained from such experimentation were found to have been reported in the literature for the macrophage system only a few months earlier.

2. METHODS

2.1 Tritiated cAMP solution

2.2 Propagation of Ehrlich ascites cells

2.3 Removal and *in vitro* incubations of Ehrlich cells

2.4 Acid extraction of Ehrlich cells

2.5 Purification of cAMP in cellular extracts

2.6 cAMP assays

2.7 *In vivo* experiments

2.8 Adenylate cyclase assays

2. METHODS

2.1 *Tritiated cAMP solution*

Adenosine-^{3}H(G)3',5'-cyclic monophosphate (24 Ci/mmole) was purchased from New England Nuclear. The radiochemical compound was contained in a 50% aqueous ethanol solution. That solution will hereafter be referred to as the stock cAMP solution. The stock solution was stored at -20°C.

The purity of the labelled cAMP was assessed by paper chromatography; 0.25 μCi of ^{3}H-cAMP was spotted on Whatman #1 chromatography paper together with the appropriate markers. The paper chromatogram was developed with 1 N ammonium acetate, pH 7: 95% ethanol (30:75). The ^{3}H-cAMP lane was cut into 3-4 cm strips. Each strip was eluted with distilled water and the aqueous eluates were counted in Aquasol scintillation cocktail (New England Nuclear). Figure 1 shows that most of the radioactivity was located in one spot coincident with cAMP. By that method, 87% of the radioactivity was recovered in the cAMP spot; 6.5% was associated with the spot corresponding to adenosine and inosine.

2.2 *Propagation of Ehrlich ascites cells*

Ehrlich ascites tumour cells were maintained by weekly intraperitoneal injection of approximately 2.5 million cells into randomly bred ICR mice. All mice utilized were healthy and 4 to 7 weeks of age. Frozen cell stocks were made available through the courtesy of Dr. J.F. Henderson (McEachern Laboratory, University of Alberta, Edmonton).

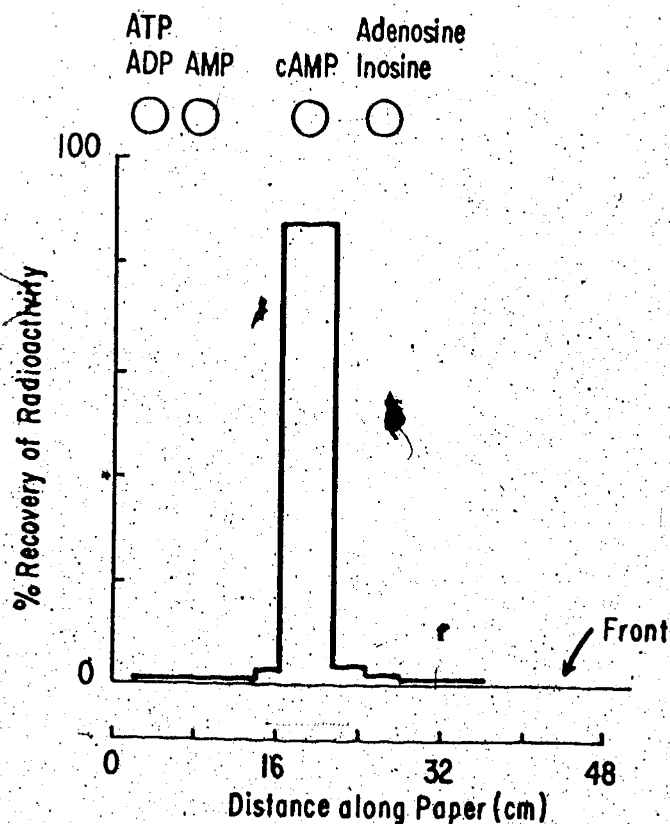


Figure 1. Purity of radiolabelled cAMP. 0.25 µCi of ^{3}H-cAMP was spotted on a 20 × 57 cm sheet of Whatman #1 chromatography paper. On adjacent lanes, 5 µl of 10 mM solutions of ATP, ADP, AMP, cAMP, adenosine and inosine were spotted as markers. The paper chromatogram was developed with 1 N ammonium acetate, pH 7:95% ethanol (30:75) for 9.5 hours at 23°. The chromatogram was dried, the markers visualized under ultraviolet light and the ^{3}H-cAMP lane cut into 3 to 4 cm strips. Each strip was then eluted with 1 ml distilled water for 1 hour at 23°. The total aqueous eluates were counted in 10 ml Aquasol scintillation cocktail in a Picker Nuclear counter. The results express the percentage of the radioactivity associated with each strip.

2.3 *Removal and in vitro incubations of Ehrlich cells*

Ehrlich ascites cells were extracted from tumour bearing mice, washed and prepared for *in vitro* incubation as previously described (Crabtree and Henderson, 1971). In short, four to six days after tumour implantation, the cells were removed from the mice and washed at least three times with cold extraction medium (140 mM NaCl; 10 mM Tris buffer, pH 7.4; 4 mM sodium phosphate buffer, pH 7.4), containing 5.5 mM glucose. Blood cells, if present, were separated from the tumour cells by repeated centrifugation. The final washed cell pellets were suspended in modified Krebs-Ringer phosphate medium (110 mM NaCl; 4.9 mM KCl; 1.2 mM $MgSO_4$; 25 mM sodium phosphate buffer, pH 7.4) containing 5.5 mM glucose and adjusted to obtain 3% (v/v) cell suspensions (approximately 8×10^6 cells/ml). The suspensions were stored on ice until the start of incubations.

All incubations were performed at 37°C in a water bath with shaking at 84 oscillations per min and air as the gas phase. A 15 min incubation period preceeded the addition of all drugs unless otherwise indicated. Plastic Erlenmeyer vessels (50 ml capacity) were utilized for the incubations. The total suspension volume did not diminish after incubation periods up to 120 min. All drugs were dissolved in saline and the total drug additions never exceeded 2% of the total suspension volume. Solutions of the catecholamines were adequately protected from heat and light and new solutions were prepared weekly. When not in use, solutions of all drugs were stored at -20°C.

l-Epinephrine bitartrate, *dl*-isoproterenol hydrochloride, *l*-phenylephrine hydrochloride and theophylline were obtained from

Sigma Chemical Company. Orciprenaline sulfate was obtained from Dr. Fröhlke (Boehringer Ingelheim, Germany) and prostaglandin E_1 from Dr. J. Pike (Upjohn Company, Kalamazoo).

2.4 *Acid extraction of Ehrlich cells*

After the desired periods of incubation, 0.5 ml aliquots of incubating cell suspensions were quickly transferred into polypropylene tubes (Eppendorf, 1.5 ml capacity) containing 0.5 ml of ice cold 0.6 M trichloroacetic acid and ^{3}H-cAMP (1800 cpm) to monitor recovery of the cyclic nucleotide. Acid extraction was allowed to proceed for at least 15 min at 4°C; the total extracts were then quickly frozen in liquid nitrogen and thawed once. Centrifugation at high speed for 2 min in a microcentrifuge (Eppendorf, 16000*g*) yielded separation of the final acid extract (approximately 1 ml) from the protein-DNA pellet. Preliminary experiments indicated that a second extraction of the pellet with 5% trichloroacetic acid did not increase the recovery of radioactivity substantially.

The precipitated protein and DNA were solubilized in 0.2 ml of 0.5 N NaOH for 90 min at 37°C or overnight at 23°C. Protein content was assayed by the method of Lowry *et al.* (1951) using purified bovine serum albumin (Sigma Chemical Company) as a standard. The content of cAMP was also determined per unit weight DNA and per cell. For DNA determinations, at least 25 million cells were extracted in order to obtain enough material to assay according to Burton's modification of Dische's diphenylamine test (Burton, 1956) using 2'-deoxyadenosine (Sigma Chemical Company) as a standard. Cell numbers

were determined using a hemacytometer after proper dilution of the tumour cell suspensions. Routinely, all of the above assays were performed in duplicate and new calibration curves were drawn for every protein and DNA assay.

The effect of *in vitro* incubations on protein and DNA content per cell was investigated. The cell numbers did not change significantly for incubation periods up to 120 min. Similarly, protein content per cell (0.425 μg/cell; range 0.405 to 0.443) and DNA concentration per cell (5.3 pg/cell; range 5.14 to 5.79) varied only within experimental error for periods up to 120 min at 37°C; the numbers represent results from 7 duplicate determinations obtained at intervals for 120 min. Therefore, all three parameters remained stable during *in vitro* incubations and can be utilized as a reference for cAMP determinations.

2.5 *Purification of cAMP in cellular extracts*

The acid extracts (about 1 ml) obtained above were extracted three times with 2 volumes of water-saturated ether. The final aqueous layer was heated at 70°C for 5 min to remove the ether.

The extracts were then applied to small columns (0.4 × 7 cm) of AG 50W-X4 cation exchange resin in the hydrogen form (Bio Rad; 100 to 200 mesh). To prepare the columns, 6 ml of a 50% aqueous suspension of the exchange resin was poured into siliconized Pasteur pipets containing glass wool to prevent passage of the resin. The columns were washed with at least 20 volumes of distilled water just prior to the addition of the cellular extracts. Following the application of

the extracts, elution was continued with distilled water. The first 4 ml of aqueous eluate were discarded; the following 4 ml were collected and constituted the cAMP fraction. Figure 2 illustrates the elution profile of an actual ether-treated tissue extract supplemented with 0.05 μmole of both ATP and AMP, and another containing 0.025 μCi ^{3}H-cAMP; AMP remained bound to the resin after elution with 15 ml distilled water. The cAMP fraction was limited to fractions (1 ml) 6 to 9 inclusive in order to eliminate impurities inasmuch as possible. Approximately 50 to 60% of the administered cAMP was routinely recovered in that fraction. The pH elution profile indicated that neutrality had been reached by fraction 3 and was maintained thereafter.

The total cAMP fraction was freeze-dried and the residue redissolved in 0.1 ml distilled water. To monitor recovery, 10 μl aliquots were counted in duplicate in 10 ml Bray's scintillation fluor (60 g purified naphthalene; 20 ml ethylene glycol; 100 ml methanol; 4 g 2,5-phenyloxazole, Fisher Scientific; and 0.2 g *p*-bis [2-(5-phenyloxazolyl)]benzene, Packard Instrument Company, were dissolved to 1 litre in 1,4-dioxane obtained from Fisher Scientific) using a Picker Nuclear scintillation counter.

2.6 *cAMP assays*

The content of cAMP was assayed in the purified extracts by the protein binding method of Gilman (1970). The method follows the principle of saturation assays (Ekins and Newman, 1970) and takes advantage of the specificity of cAMP binding proteins, which presum-

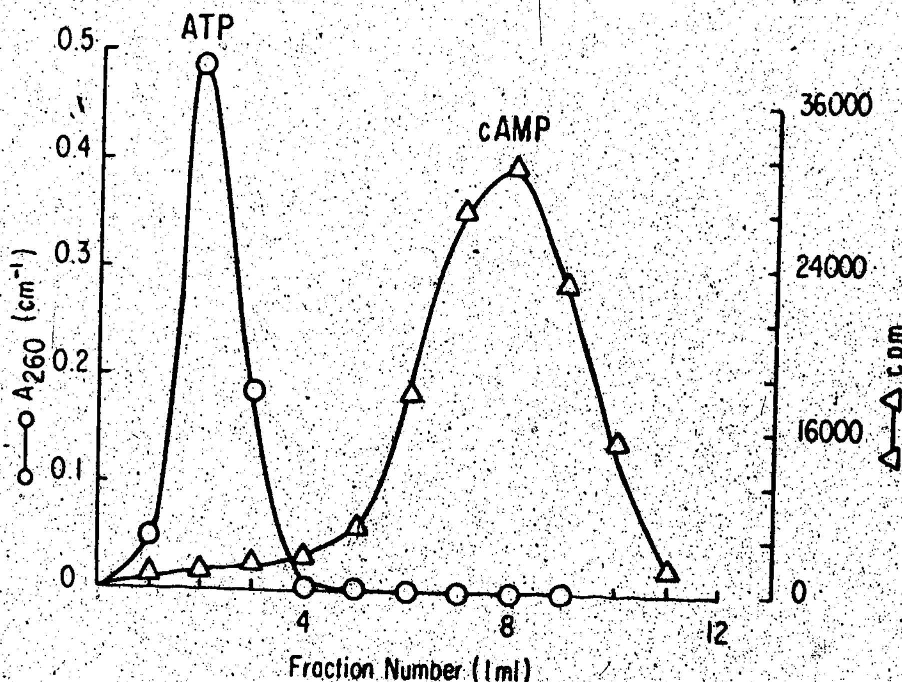


Figure 2. Elution of ^{3}H-cAMP and ATP from AG 50W-X4 cation exchange resin. Two acid extracts were prepared from Ehrlich cells as described under section 2.4; one was then supplemented with 0.05 μmole of both ATP and AMP and the other with 0.025 μCi ^{3}H-cAMP. The acid extracts were extracted three times with two volumes of water saturated ether and the final aqueous layer was heated at 70° for 5 min. The extracts were then applied to small columns (0.4 × 7 cm) of AG 50W-X4 cation exchange resin in the hydrogen form (Bio Rad; 100 to 200 mesh) and the columns were eluted with water. Fractions (1 ml) were collected and the absorbance at 260 nm or the radioactivity present was determined; the applied extracts (1 ml) constituted fraction #1. For the absorbance measurements, the fractions were diluted to 10 ml with distilled water; for radioactive counting, whole fractions were counted in 10 ml Bray's scintillation medium. AMP remained bound to the resin after elution with 15 ml distilled water.

ably constitute the regulatory subunits of cAMP dependent protein kinases.

The method of Gilman requires the preparation of two protein fractions from beef muscle: the binding protein and the "inhibitor protein". The binding protein was prepared from beef heart essentially following the method of Miyamoto *et al.* (1969), as described for brain protein kinase, through the acid and ammonium sulfate precipitation steps. In short, fresh beef hearts were transported on ice from a nearby slaughter house, dissected from the surrounding fat tissue and the ventricles cut into small cubes. All subsequent steps were performed in a cold room at 4°C. The tissue was homogenized in a Waring blender in 3 volumes of cold 4 mM EDTA (Fisher Scientific), pH 7. The slurry was then centrifuged at 12000*g* for 15 min at 4°C. Acetic acid (1 N) was added dropwise to the supernatant until a pH of 4.9 was reached. After centrifugation at 12000*g* for 15 min, the pH of the supernatant was readjusted to 7 using 1 M potassium phosphate buffer, pH 7.2. Ultra pure, enzyme grade ammonium sulfate (Schwarz/Mann, Montreal) was then added slowly to obtain a 32.5% (w/v) salt solution and the slurry stirred at 4°C for 20 min. Following centrifugation at 12000*g* for 15 min, the supernatant was discarded and the precipitate redissolved in 200 ml of 5 mM potassium phosphate buffer, pH 7, containing 2 mM EDTA. The 32.5% salt extract was then dialyzed at 4°C against 30 volumes of the same buffer for 15 hours with one change of buffer. Any precipitate was then removed by centrifugation at 12000*g* for 15 min. The preparation was then subjected to DEAE-cellulose chromatography as described by Gilman (1970).

SERVA DEAE cellulose (0.7 mequ/g dry weight) was swollen in distilled water for 12 hours, washed with 0.5 N NaOH for 30 min and then with water until the supernatant fluids were neutral. The cellulose was then washed with 0.5 N HCl for 30 min followed by distilled water until neutral. A 3 × 40 cm column was poured with the washed cellulose and equilibrated with 5 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA.

The column was then loaded with 690 OD_{280} of enzyme (30 ml of dialyzed ammonium sulfate preparation). Figure 3 illustrates the DEAE purification of the cardiac binding protein. The column was washed with 5 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA until the first visibly yellow protein peak was eluted. The flow rate was 1.3 ml/min throughout and 10 ml fractions were collected. Elution was then continued with 100 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA from fraction 22 until fraction 49 when the newly appearing protein peak began to decline. Elution was continued with 300 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA from fraction 50 until fraction 100. The absorbance at 280 nm was determined for alternate fractions (Figure 3).

Aliquots of alternate fractions were also assayed for cAMP binding activity (50 mM sodium acetate, pH 4; 1 pmole ^{3}H-cAMP, 20000 cpm; 10 µl of fractions; total volume 0.1 ml; after equilibration for 1 hour at 4°C, assay samples were filtered and counted as described by Gilman (1970)). The cAMP binding profile is also illustrated in Figure 3. Two main binding peaks are evident, namely peak 1 (fractions 39 to 44) and peak 2 (fractions 67 to 69).

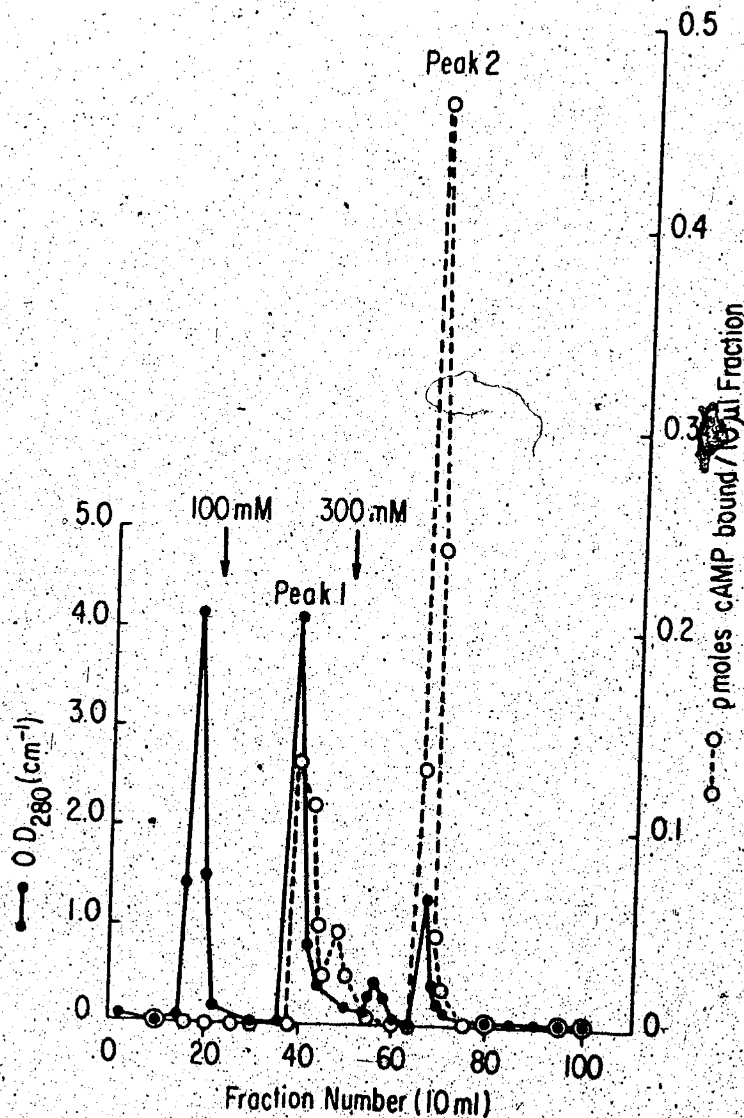


Figure 3. DEAE-Purification of cardiac binding protein. A 3 × 40 cm SERVA DEAE cellulose column was poured and equilibrated with 5 mM potassium phosphate buffer, pH 7, containing 2 mM EDTA. The column was loaded with 690 OD_{280} of heart binding protein preparation processed through the acid and salt precipitation steps as described in the text. The column was washed with 5mM potassium phosphate buffer (pH 7) containing 2 mM EDTA until fraction 21. The flow rate was 1.3 ml per min throughout and 10 ml fractions were collected. Elution was then continued with 100 mM buffer containing 2 mM EDTA until fraction 49 when the newly appearing protein peak began to decline. Elution thereafter was continued with 300 mM buffer containing 2 mM EDTA until fraction 100. The absorbance at 280 nm was determined for alternate fractions. Aliquots of alternate fractions were also assayed in duplicate for cAMP binding activity [50 mM sodium acetate, pH 4; 1 pmole ^{3}H-cAMP, 20,000 cpm; 10 μl of alternate fraction; total volume 0.1 ml; after equilibration for 60 min at 4°, assay samples were filtered and counted as described by Gilman (1970)]

Protein kinase activity was also determined using 10 μl aliquots of alternate fractions; the assay was carried out as previously described (Eckstein *et al.*,1974) using histone II (Sigma Chemical Company) as substrate (50 mM sodium acetate, pH 6.2; 10 mM $MgCl_2$; 0.3 mM EGTA; 0.2 mM EDTA; 1 mg/ml histone type II; 2 mM [γ-^{32}P] ATP was obtained from International Chemical Nuclear, California. Total reaction volume was 0.1 ml. Incubations were carried out for 10 min at 30°C. The reaction was terminated and processed by the filter paper precipitation technique described by Reimann *et al.* (1971). Protein kinase activity exhibited the same profile as cAMP binding; basal protein kinase activity from fraction 40 (peak 1) was 13.25 pmoles ^{32}P transferred/min/10μl and was stimulated approximately 3 fold by 10^{-6}M cAMP. Protein kinase activity from fraction 67 (peak 2) was 13.74 pmoles ^{32}P transferred/min/10μl and was stimulated 5 fold in the presence of 10^{-6} M cAMP. Therefore, cAMP binding correlated well with cAMP-stimulated protein kinase activity.

Binding protein was precipitated from fractions 67 to 69 by the slow addition of solid ammonium sulfate (35 g/100 ml). After stirring for 20 min at 4°C, the slurry was centrifuged at 12000*g* for 15 min. The precipitate was redissolved in a minimal volume of 5 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA, and dialyzed against 1 l of the same buffer for 8 hours with one change of buffer. The protein concentration of the dialysate was adjusted to 1 mg/ml; protein determination was performed by the method of Lowry *et al.* (1951). Aliquots (1 ml) of the final binding protein preparation were stored under liquid nitrogen.

Gilman recommends the use of an "inhibitor protein" in cAMP

determinations by the protein binding method. Although the protein is not necessary in the assay, it does add sensitivity to the method. This protein fraction was prepared from beef skeletal muscle following the procedure of Appleman *et al* (1966).

For cAMP determinations, the following were contained in a total volume of 0.1 ml: 50 mM sodium acetate, pH 4; 19 μg inhibitor protein; 1 μg cardiac binding protein and 0.2 pmole ^{3}H-cAMP (4000 cpm). To prepare the calibration curves, quantities of cAMP ranging from 0.5 to 10 pmoles were added in addition to the above; the exact concentration of the cAMP solutions utilized for calibration purposes was determined spectrophotometrically (ϵ_{260} = 15000 l/mole·cm). Experimental tubes contained 10 to 20 μl of purified tissue extracts which usually contained 0.5 to 5 pmoles cAMP. The reaction mixtures were contained in small polypropylene reaction tubes (Eppendorf, 1.5 ml capacity). The assay tubes were incubated at 4°C for 60 to 90 min; 1 ml [illegible]M potassium phosphate buffer (pH 6) was then added to t[illegible]ts which were immediately filtered through membrane filte[illegible]cron pores; Matheson-Higgins Co., Inc., Woburn). Two additi[illegible]es of the tubes were similarly subjected to filtration through [illegible]ame filter and the filter was then washed two times with 4 ml al[illegible] of the same buffer. The steps of filtering and washing were [illegible]ried out rapidly, one assay tube at a time.

[illegible]he filters were then dissolved in a medium containing 5 ml toluene, [illegible].8 ml ethylene glycol monomethyl ether, 25 mg 2,5-diphenyloxazole and 1 mg 1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene and counted in a Picker Nuclear scintillation counter at an efficiency of approximately 30% as determined by the external standard method. The

efficiency for samples from the same experiment did not vary by more than 1%. Blanks conducted in the absence of binding protein yielded no detectable binding of radioactivity to the filters. Counts per 10 min were plotted against the cAMP content of calibration samples on a double logarithmic plot. Figure 4 illustrates a typical cAMP assay calibration curve. The calibration curve yielded a straight line in the range of 0.5 to 10 pmoles cAMP. However, the line was bent at lower cAMP concentrations. The cAMP content of experimental samples was usually in the straight line segment of the calibration curve. All determinations were done in duplicate and a new calibration curve was drawn for every experiment. Preliminary experiments indicated that the addition of 50 pmoles of AMP or ATP to cAMP assay mixtures had no effect on the cAMP calibration curve between 0.5 and 5 pmoles.

In order to test whether the purified cell extracts utilized for cAMP determinations contained impurities which interfered with cyclic nucleotide binding, 10 µl aliquots of purified cAMP extracts were treated with cyclic nucleotide phosphodiesterase. Cyclic nucleotide phosphodiesterase was prepared from hog brain according to the procedure described by Nair (1966) for dog heart phosphodiesterase (prepared by S.G. McKenzie in this laboratory). The phosphodiesterase reaction mixtures contained in a volume of 20 µl: 5 mM Tris buffer, pH 7.5; 1 mM $MgCl_2$; 3 µg phosphodiesterase and 10 µl of purified cAMP extracts. Reactions were carried out at 37°C for 30 min and were terminated by boiling for 3 min in a water bath. Preliminary experiments with radioactive substrate indicated that such treatment hydrolyzed better than 95% of the cAMP present. The phosphodiesterase reaction

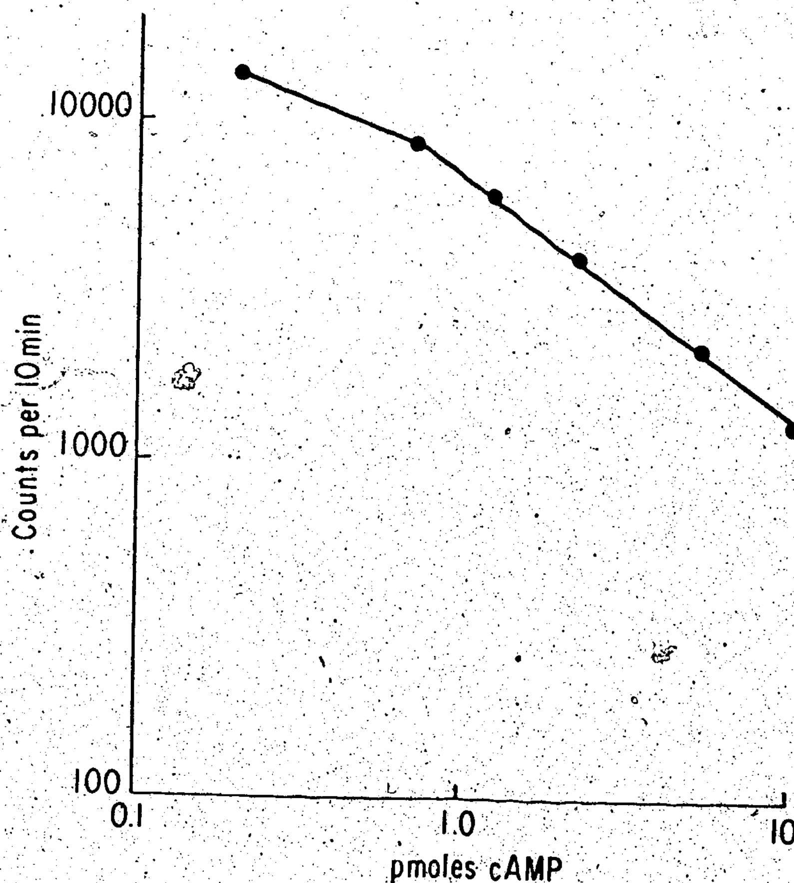


Figure 4. cAMP assay calibration curve. Counts per 10 min are plotted against the cAMP content of calibration samples on a double logarithmic plot to obtain a straight line. Assay samples contained in a total volume of 0.1 ml: 50 mM sodium acetate, pH 4; 19 µg inhibitor protein; 1 µg cardiac binding protein; 0.2 pmoles ^{3}H-cAMP (4000 cpm) and 0.5 to 10 pmoles unlabelled cAMP. After incubation at 4° for at least 60 min, assay samples were filtered and the filters were processed for counting as described in the text.

mixtures were freeze-dried and the residues were subjected to cAMP determination by the protein binding method in the presence of known amounts of unlabelled cAMP. Controls were conducted in which aliquots of cellular extracts were replaced by distilled water in the phosphodiesterase reaction mixtures. Such experiments proved to satisfaction that no impurities in the cAMP extracts interfered with cAMP binding to the cardiac protein preparation over the range of cAMP concentrations of a calibration curve. Experimental samples treated with phosphodiesterase were used as blanks occasionally to assure the authenticity of cAMP measurements.

2.7 *In vivo experiments*

The effects of drugs on cAMP levels in Ehrlich cells within the peritoneal cavity of mice were investigated. Drugs were introduced within the peritoneal cavity of the mice in a total volume of 0.2 ml saline. After the desired periods of time, the tumour cells were removed from the host, plunged into ice cold trichloroacetic acid and purified cAMP extracts were prepared as described above. The amount of biological material (cells) extracted was assessed by duplicate DNA determinations.

Since the catecholamines stimulate adenylate cyclase from Ehrlich cells (Bär and Henderson, 1972) and since handling of the animals prior to and during cell removal might cause adrenal medullary discharge of catecholamines, the effect on cAMP levels of the method employed to sacrifice mice was investigated. Furthermore, two methods for tumour cell removal were investigated with respect to a possible

effect on cellular cAMP content. The results are summarized in Table 1. There were no major differences in tumour cell cAMP content whether the mice were sacrificed by cervical fracture, decapitation or whether they were anesthetized with pentobarbitol (150 mg/kg). The two methods of extraction were the following:

Method A - 0.1 ml of concentrated cell suspension in ascites fluid was withdrawn using an Eppendorf pipette (0.1 ml) and quickly plunged into 0.9 ml ice cold 0.6 M trichloroacetic acid as soon as possible after the sacrifice of the animal (about 15 sec).

Method B - 2 ml of cold saline was quickly injected into the peritoneal cavity of the sacrificed mouse; 0.5 ml of cell suspension was then withdrawn and quickly added to 0.5 ml of cold 1.2 M trichloroacetic acid. The data presented in Table 1 illustrate that the method of extraction did not drastically influence the cAMP content of the tumour cells.

For routine *in vivo* work, the mice were sacrificed by cervical fracture, the abdominal wall and peritoneum pierced with scissors and 0.1 ml of cell suspension in ascites fluid was quickly withdrawn with an Eppendorf pipette. The cells were immediately plunged into 0.9 ml of cold 0.6 M trichloroacetic acid; the acid extracts were processed as described previously to obtain cAMP content per unit weight DNA. Preliminary experiments indicated that ascites fluid (0.1 ml, 3000*g* supernatant) from mice bearing 7 day old tumours contained no detectable cAMP or DNA when processed in an identical way to the ascites cell suspensions. Thus, ascites fluid did not interfere with the determinations of cAMP content within Ehrlich cells.

TABLE 1

THE EFFECT OF THE METHOD OF KILLING AND TUMOUR EXTRACTION ON cAMP CONTENT IN EHRLICH CELLS

Method of Killing	Method of Extraction *	cAMP Content ±S.E. pmoles cAMP/μg DNA (n)**
DECAPITATION	A	0.233±0.034 (3)
DECAPITATION	B	0.167±0.037 (2)
CERVICAL FRACTURE	A	0.183±0.012 (3)
PENTOBARBITOL ANESTHESIA***	A	0.176±0.005 (3)
ANESTHESIA, THEN DECAPITATION	A	0.165±0.004 (2)

* Refer to text for description of extraction methods A and B.

** n=number of tumours (mice) extracted for each determination.

*** Mice were anesthetized with pentobarbitol administered i.p. (150 mg/kg); the tumour cells were extracted 5 min after the loss of the blinking reflex.

2.8 *Adenylate cyclase assays*

Adenylate cyclase assays were performed using membrane preparations from cells incubated as described in section 2.3. Following *in vitro* incubations in the presence or absence of drugs for periods of time specified for the individual experiments, 10 ml of 3% (v/v) Ehrlich cell suspensions were diluted with 3 volumes of ice cold modified Krebs-Ringer phosphate medium (section 2.3) and immediately centrifuged at 1500*g* for 5 min at 4°C. The cells were then washed with 10 ml of cold medium and centrifuged as before. The washed cells were resuspended in 3 ml of cold distilled water and allowed to swell for 5 min on ice (Bär and Henderson, 1972). Subsequently, the cells were poured into a chilled homogenizer tube containing 0.3 ml of 100 mM Tris (pH 7.5) and 10 mM $MgCl_2$. The mixture was homogenized immediately with a tight fitting Teflon pestle. The homogenate was centrifuged at 1500*g* for 10 min at 4°C. The resulting pellet was washed with 5 ml of cold 10 mM Tris (pH 7.5) and 1 mM $MgCl_2$ and centrifuged as before; the latter step was repeated and the final pellet containing adenylate cyclase was resuspended in approximately 1 ml of the same buffer. Basal and stimulated enzyme activity was assayed immediately after preparation.

The standard adenylate cyclase assays were essentially performed as previously described (Bär and Hechter, 1969). The assays contained, in a total of 0.05 ml, 40 mM Tris (pH 8), 5 mM $MgCl_2$, 0.1% bovine serum albumin, 10 mM sodium creatine phosphate (Boehringer-Mannheim, New York), 1 mg/ml creatine kinase (Boehringer-Mannheim), 0.5 mM sodium cAMP and 0.1 mM [α-^{32}P]ATP (International Chemical Nuc-

lear, California). Enzyme protein was added to start the incubations which were carried out at 37°C for 20 min. The reactions were stopped by the addition of 5 µl of a solution containing 0.125 M EDTA and 0.05 M each of cAMP, ATP and 5'-AMP, and the reaction tubes quickly stored on ice. Further processing and chromatography on polyethyleneimine-impregnated cellulose thin layer plates (Macherey-Nagel and Co., Germany) were carried out as recently described (Bär, 1975). Adenylate cyclase activity is expressed as pmoles cAMP formed per mg protein per min; protein determinations were conducted according to the method of Lowry *et al.* for insoluble protein (Lowry *et al.*, 1951).

3. RESULTS AND DISCUSSION

3.1 Time course of elevation of cAMP following epinephrine *in vitro*

3.2 Absence of extracellular accumulation of cAMP

3.3 Pharmacological properties of the catecholamine receptor in Ehrlich cells

3.4 Time course of elevation of cAMP following prostaglandin E_1 *in vitro*

3.5 Dose-response behaviour to epinephrine and prostaglandin E_1

3.6 Effect of a second addition of hormone

3.7 Search for inhibitory activity possibly associated with incubation medium

3.8 Effect of propranolol added after epinephrine

3.9 Effect of theophylline added after epinephrine

3.10 Partial refractoriness of stimulated cells to the effects of epinephrine

3.11 Hormone-dependent modification of adenylate cyclase

3.12 A proposed mechanism for hormone-induced desensitization of adenylate cyclase

3.13 Hormonal stimulation of Ehrlich cells *in vivo*

3.14 Time course of the cAMP response of Ehrlich cells to epinephrine *in vivo*

3. RESULTS AND DISCUSSION

3.1 *Time course of elevation of cAMP following epinephrine in vitro*

Ehrlich ascites tumour cells previously incubated for 15 min at 37°C responded to epinephrine (10^{-6} M) with a very rapid and pronounced increase of cAMP levels, followed by a very quick quick decline toward basal levels despite the continued presence of hormone (Figure 5). Results from 10 independent experiments indicated that cAMP was increased 6 to 24 fold above basal levels after 1 min and 2.4 to 5 fold after 10 min; results from 5 experiments showed 1.8 to 2.6 fold increases after 60 min. Basal cAMP levels varied between 0.9 and 3.6 × 10^{-18} moles/cell in 13 determinations following incubation of isolated cells for 15 min at 37°C; the mean value was 1.8 × 10^{-18} moles/cell. Basal cAMP content did not change noticeably for incubation periods up to 120 min at 37°C, as monitored over 3 independent time course studies.

The broad range of the magnitude of increase of cAMP content following stimulation by the same concentration of hormone can be due to several factors. We have not investigated this question but believe that there may be variable hormonal sensitivity of different tumour cell batches, and that the humoral state of the animals bearing the tumours, with special reference to the circulating catecholamines, may be a determinant. The basal activity and hormonal sensitivity of adenylate cyclase prepared from Ehrlich cells has also been found to vary over a similarly wide range for different batches of cells (Bär and Henderson, 1972). As a consequence of this variabil-

ity in the response of different tumours to hormone, data obtained from different experiments have not been pooled.

Figure 5 illustrates a typical *in vitro* response of tumour cells to stimulation by 10^{-6} M epinephrine in the presence and absence of 1 mM theophylline. Theophylline greatly enhanced the response to the hormone suggesting a rapid turnover rate of cAMP following hormonal stimulation. Theophylline (1 mM) alone caused a 2 to 2.5 fold increase in cAMP after 5 min and these elevated levels were maintained until at least 120 min at 37°C. Therefore, phosphodiesterase is an important determinant of cAMP levels in Ehrlich cells.

It is of special interest to note that the time function in the presence of 1 mM theophylline is qualitatively similar to the time course in the presence of hormone alone (Figure 5); the response to 10^{-6} M epinephrine was enhanced 2.5 fold at 1 min, 3.5 fold at 10 min and 3.7 fold at 60 min when performed in the presence of 1 mM theophylline. Therefore, the secondary decline of cAMP cannot be accounted for by a mechanism involving only cyclic nucleotide phosphodiesterase, unless the particular phosphodiesterase involved is resistant to, or protected from, inhibition by theophylline. If the secondary decline was the result of a mechanism involving a phosphodiesterase subject to inhibition by theophylline, qualitative aspects of the time course would certainly be drastically altered in the presence of a phosphodiesterase inhibitor. The observed effect of theophylline on the time course would be expected if the phosphodiesterase inhibitor simply decreased the rate of destruction of cAMP to approximately the same extent throughout the entire time course.

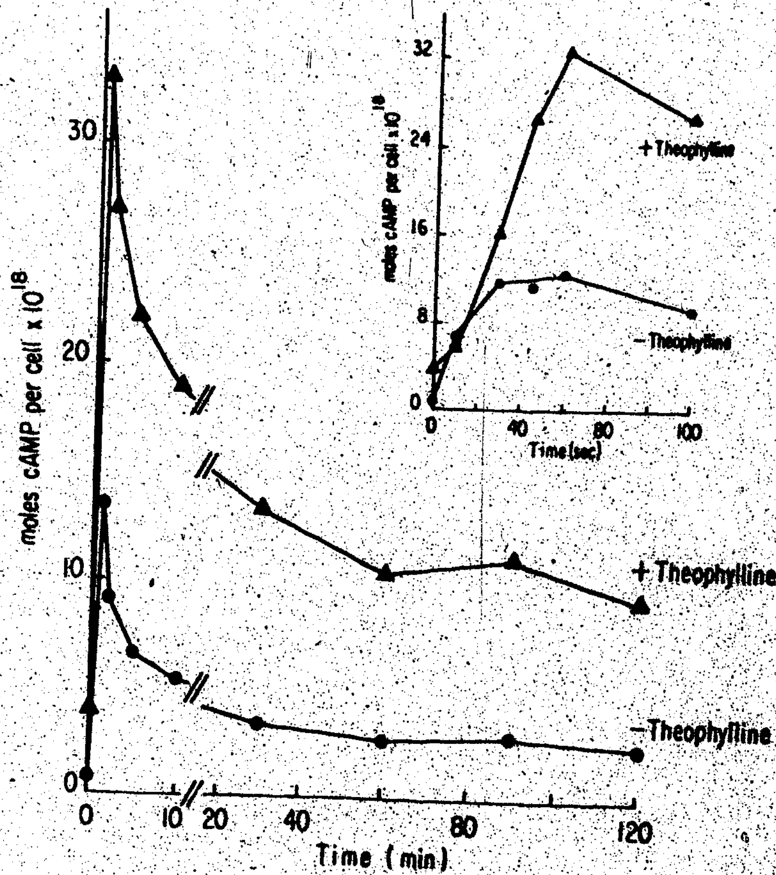


Figure 5. Time course of the cAMP response to 10^{-6}M epinephrine. Ehrlich cell suspensions (3%, v/v) were incubated under standard conditions in the presence or absence of 1 mM theophylline for 15 min; epinephrine was then added (0 time) to obtain a final concentration of 10^{-6}M. Aliquots (0.5 ml) were withdrawn at intervals and acid extracts were prepared and processed for cAMP determination. Results are expressed as moles × 10^{-18} cAMP per cell. Cell number was determined in duplicate using a hemacytometer. The inset demonstrates the time course of cAMP elevation for the first 2 min following the addition of epinephrine.

The inset to Figure 5 displays the time course of cAMP elevation during the first 2 min following stimulation by 10^{-6} M epinephrine in the presence and absence of 1 mM theophylline. It is evident from the inset that the true maximum was indeed reached at about 1 min following the addition of hormone. Qualitatively identical results to those illustrated in Figure 5 were obtained in 5 studies with other batches of Ehrlich cells.

The dependence of the time course on the epinephrine concentration was investigated. Time courses were qualitatively identical over a 1000 fold range of epinephrine concentrations (10^{-7} to 10^{-4} M); Figure 6 illustrates results for 10^{-7} M and 10^{-5} M epinephrine. These results do not favour the possibility that ATP depletion may account for the secondary decline in Ehrlich cells since at high doses of hormone ATP should be depleted more rapidly and thus the peak response and the phase of decline of cAMP levels would occur at an earlier point in time.

Several parameters were studied that might influence the effects of epinephrine on cAMP levels in Ehrlich cells. As mentioned earlier, DNA and protein content per cell remained fairly constant during *in vitro* incubations; thus, time courses were identical whether cAMP content was expressed per unit weight DNA, acid precipitable protein or per cell. Furthermore, the duration of the preincubation of cells for periods up to 60 min did not affect the magnitude of the response to hormone or the qualitative aspects of the time course. Hence, the passive release of a hormone antagonist into the incubation medium seems improbable.

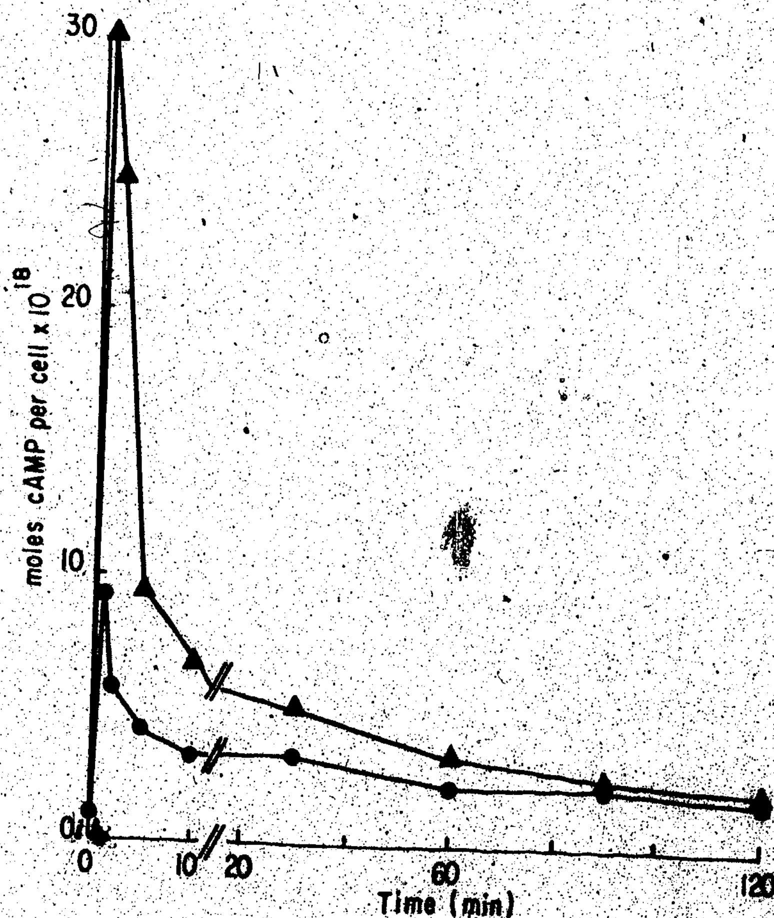


Figure 6. Time course of cAMP response to 10^{-5} and 10^{-7}M epinephrine. Ehrlich cell suspensions (3%, v/v) were incubated under standard conditions in the presence of 10^{-5}M (closed triangles) or 10^{-7}M (closed circles) epinephrine for periods up to 120 min. A 15 min incubation period preceded the addition of hormone (0 time). Aliquots were withdrawn at intervals for cAMP determination. Cell number was determined in duplicate using a hemacytometer.

It was necessary to prove that the observed time course was not peculiar to the standard incubation medium employed. A time course follo[illegible]timulation by 10^{-6} M epinephrine was thus performed *in vitro* usi[illegible]spended in the 15000*g* supernatant of undiluted, fresh ascite[illegible]ated from tumour bearing mice. Figure 7 demonstr[illegible] tha[illegible] experiment performed in ascites fluid showed result[illegible]ila[illegible]hose performed under standard conditions. This experim[illegible]fu[illegible] suggested that ascites fluid was free of agents which co[illegible]fere with or antagonize epinephrine stimulation of Ehrlich c[illegible]

3.2 *Absenc[illegible] extracellular accumulation of cAMP*

Th[illegible]ssibility of extracellular accumulation of cAMP was investigated [illegible]time intervals for 120 min following the addition of 10^{-6} M epinephr[illegible]. Aliquots (0.5 ml) of cell suspension were withdrawn at the [illegible] times, centrifuged for 0.5 min in a Microcentrifuge (Eppend[illegible]*g*) and the cell-free supernatants were added immediately to [illegible]5 ml of cold 0.6 M trichloroacetic acid. The acid extracts of the supernatants were processed for routine cAMP determinations. There was no detectable cAMP at any time in volumes of supernatant equivalent to those employed for routine cAMP determination in cell suspensions. Hence, values of cAMP content in our studies indeed represent intracellular cAMP; any changes observed must be due to intracellular mechanisms governing the intracellular distribution and the synthesis and breakdown of the cyclic nucleotide. It is conceivable, however, that cAMP leaking out of cells could be hydrolized

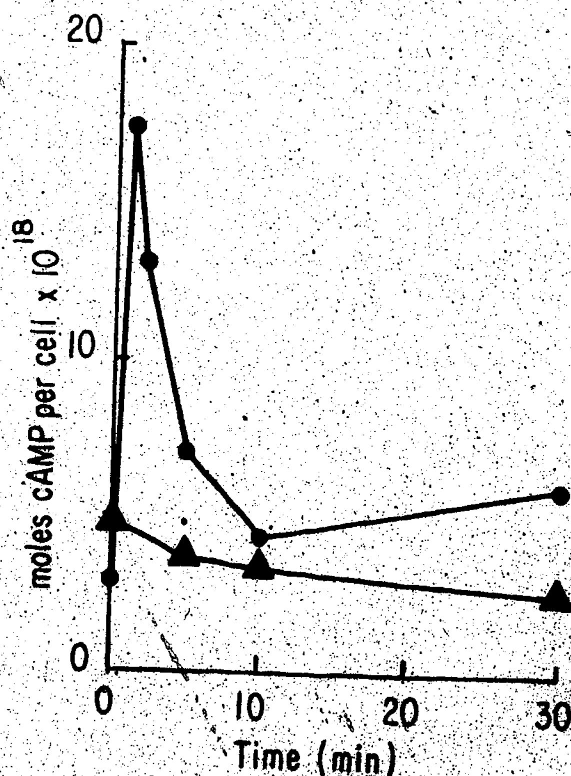


Figure 7. Time course of cAMP response to epinephrine of Ehrlich cells suspended in ascites fluid. Ehrlich cells were suspended in the 15000*g* supernatant of fresh, undiluted ascites fluid extracted from mice bearing 7 day old tumours to obtain a 3% (v/v) cell suspension. After incubation for 15 min at 37°, an aliquot of this cell suspension received epinephrine (0 time) to obtain a final concentration of 10^{-5}M (closed circles); an equal volume of saline was added to a control suspension (closed triangles). Aliquots were withdrawn at intervals for cAMP determination. Cell number was determined in duplicate using a hemacytometer. Results are from a single experiment.

rapidly by phosphodiesterases accessible to extracellular substrate, but this possibility was not investigated in the present study.

3.3 *Pharmacological properties of the catecholamine receptor in Ehrlich cells*

Propranolol (10^{-5} M) present in a 10 fold excess of the epinephrine concentration (10^{-6} M) completely antagonized the effect of hormone throughout the entire time course (Figure 8A); however, propranolol present alone did not influence basal cAMP levels. Phentolamine (10^{-5} M) had little effect on the response to 10^{-6} M epinephrine (Figure 8B). These results agree with those obtained using adenylate cyclase membrane preparations from Ehrlich cells (Bär and Henderson, 1972). Furthermore, orciprenaline (10^{-6} M) did not significantly increase cAMP levels in Ehrlich cells (Figure 8C). Since orciprenaline preferentially stimulates β_2-adrenergic receptors, this result suggests that Ehrlich cells contain receptors closely related to those of β_1-type systems.

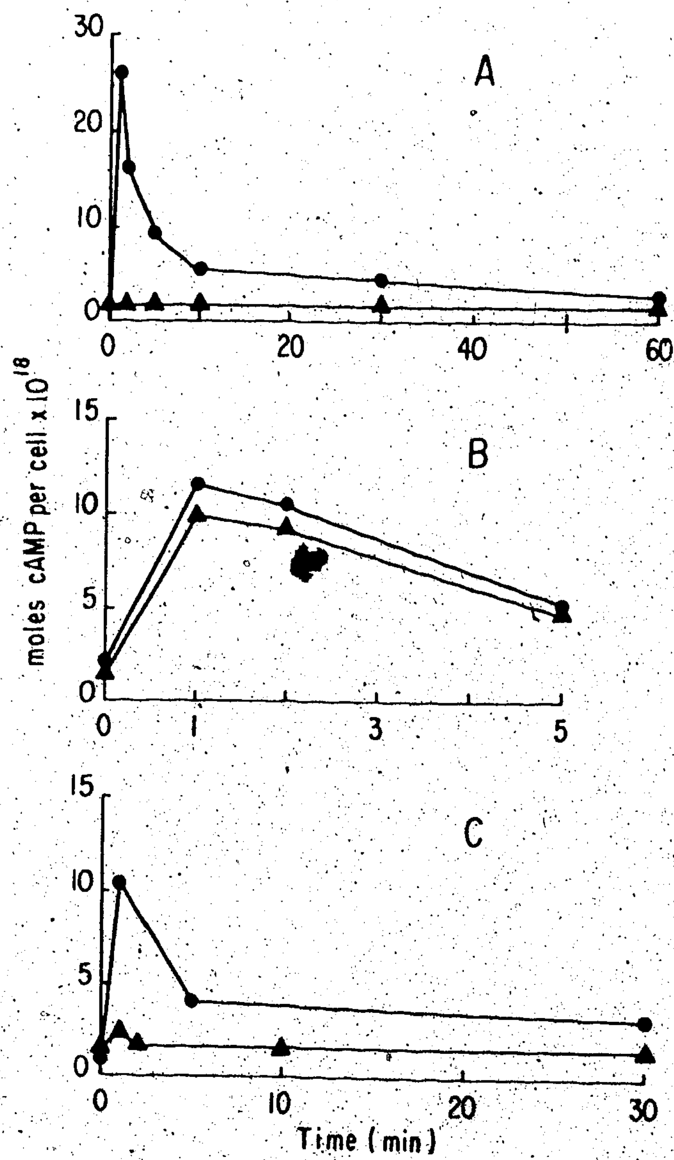
3.4 *Time course of elevation of cAMP following prostaglandin E_1 in vitro*

The time course of the elevation of cAMP content was investigated following the addition of prostaglandin E_1 ([illegible].9 × 10^{-5} M) and is displayed in Figure 9. It is evident that the rapid initial rise and subsequent decline that characterize the response to epinephrine also apply to prostaglandin E_1. However, prostaglandin E_1 was not nearly as effective as epinephrine with respect to the magnitude of the peak response of cAMP content. Results from two independent stud-

Figure 8A. Effect of propranolol on the cAMP response to epinephrine. Epinephrine (10^{-6}M) was added (0 time) to Ehrlich cell suspensions (3%, v/v) previously incubated under standard conditions in the presence (closed triangles) or absence (closed circles) of 10^{-5}M propranolol for 15 min. At intervals, aliquots (0.5 ml) were withdrawn and cAMP content was determined. Cell number was determined in duplicate using a hemacytometer.

Figure 8B. Effect of phentolamine on the cAMP response to epinephrine. Epinephrine (10^{-6}M) was added (0 time) to Ehrlich cell suspensions (3%, v/v) previously incubated under standard conditions in the presence (closed triangles) or absence (closed circles) of 10^{-5}M phentolamine. Subsequent steps were performed as described for Figure 8A.

Figure 8C. Time course of the cAMP response to orciprenaline. Ehrlich cell suspensions (3%,v/v) were incubated under standard conditions for 15 min. Orciprenaline (10^{-6}M; closed triangles) or epinephrine (10^{-6}M; closed circles) was then added (0 time) and 0.5 ml aliquots were withdrawn at intervals for the determination of cAMP content. Cell number was determined in duplicate using a hemacytometer.


A
B
C
moles cAMP per cell x 10^{18}
Time (min)

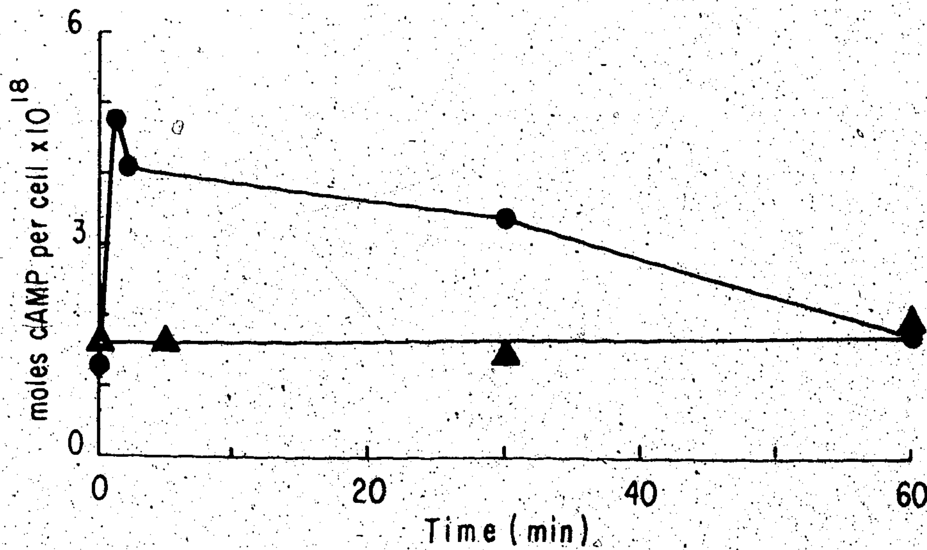


Figure 9. Time course of cAMP response to prostaglandin E_1. Ehrlich cell suspensions (3%, v/v) were incubated under standard conditions for 15 min; prostaglandin E_1 was then added to one suspension (0 time) to obtain a final concentration of 2.9×10^{-5}M (closed circles). A control suspension received an equivalent volume of saline (closed triangles). Aliquots (0.5 ml) were withdrawn at intervals and acid extracts prepared for cAMP determination. Results are from a single experiment

ies after 2.9×10^{-5} M prostaglandin E_1 indicated 3.9 and 3.6 fold increases above basal levels at 1 min; the respective increases were 2.3 and 2.8 fold after 10 min. These results suggest that a common mechanism is activated to account for the secondary decline following stimulation by epinephrine and prostaglandin E_1. Furthermore, the same 1 min lag period preceeded the initiation of the secondary decline after stimulation by prostaglandin E_1.

3.5 *Dose-response behaviour to epinephrine and prostaglandin E_1*

Dose-response relationships for epinephrine and prostaglandin E_1 are displayed in Figures 10A and 10B, respectively. The responses were determined 1 and 10 min following the addition of hormone. The elevation of cAMP content was dose dependent between 10^{-7} M and 10^{-4} M epinephrine. Four independent dose-response determinations to epinephrine yielded sigmoid curves similar to that illustrated in Figure 10A; no response was observed with 10^{-8} M epinephrine at 1 or 10 min in three of the four determinations. Maximal responses at 1 and 10 min were reached with 10^{-5} M epinephrine in three experiments; in a fourth, the maximum was observed with 10^{-6} M epinephrine. As expected, the responses to epinephrine after 10 min were less marked than at 1 min, but exhibited a similar dependence on epinephrine concentration (Figure 10A).

The response to prostaglandin E_1 was also dose dependent although no typical sigmoid pattern was demonstrable (Figure 10B); however, the highest concentration tested was 2.9×10^{-5} M. The cAMP levels were not influenced by concentrations of prostaglandin E_1 lower

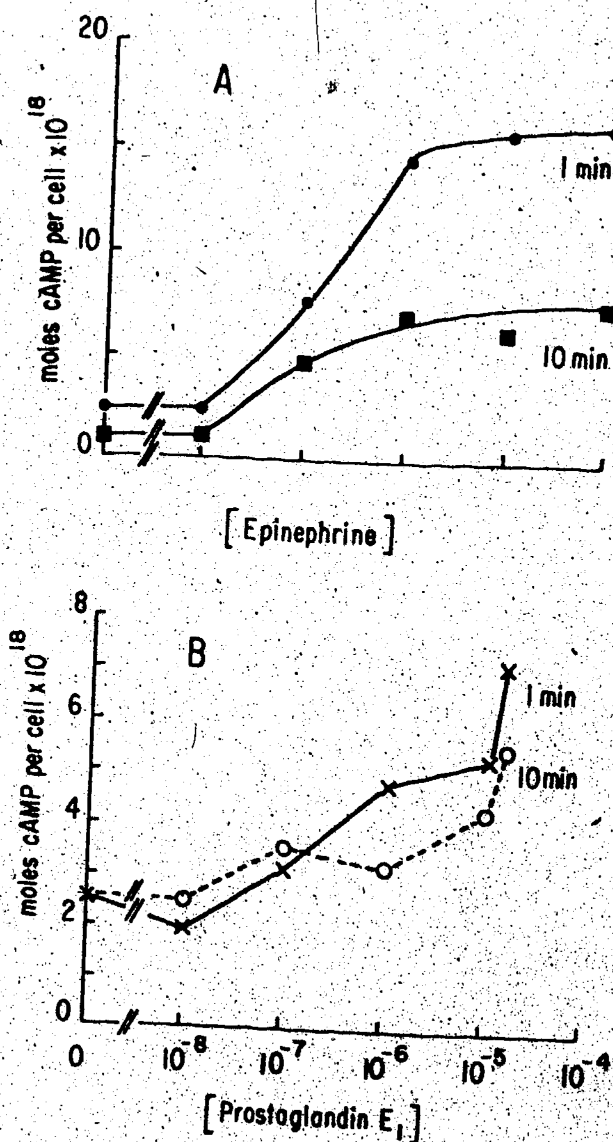


Figure 10A. Dose-response behaviour of cAMP response to epinephrine. Aliquots (3 ml) of an Ehrlich cell suspension (3%, v/v) were incubated for 15 min under standard conditions. Epinephrine was then added (0 time) to obtain doses ranging from nil to $10^{-[illegible]}$ M and aliquots were withdrawn after 1 and 10 min for the determination of cAMP content. Results are from a single experiment.

Figure 10B. Dose-response behaviour of cAMP response to prostaglandin E_1. Doses of prostaglandin E_1 ranging from nil to 2.9×10^{-5} M were tested for their effect on cAMP content in Ehrlich cells after 1 and 10 min. The experiment was performed as described for epinephrine in Figure 10A.

than 10^{-6} M. Concentrations of 10^{-4} M or higher were not tested due to their physiological irrelevance. The increase of the response observed between 1×10^{-5} M and 2.9×10^{-5} M prostaglandin E_1 (Figure 10B) was confirmed in a further study. In two additional experiments, it was found that combined doses of epinephrine (10^{-4} M) and prostaglandin E_1 (2.9×10^{-5} M) did not produce additive responses. Both the high doses of prostaglandin E_1 necessary to cause a response and the lack of additivity of hormonal effects agree with studies on adenylate cyclase prepared from Ehrlich cells (Bär and Henderson, 1972).

3.6 *Effect of a second addition of hormone*

In order to test whether the decline in cAMP was caused by destruction of hormone, a second addition of epinephrine (10^{-6} M) was made to an incubation vessel containing cells preincubated with 10^{-6} M epinephrine for 10 min (Figure 11). That addition produced just a slight increase in cAMP content indicating that the fall in cAMP cannot be explained by destruction of hormone but by resistance or partial refractoriness to the hormone present. This finding is confirmed by additional experiments described in the following section.

3.7 *Search for inhibitory activity possibly associated with incubation medium*

The incubation medium from epinephrine-treated cell suspensions was tested for the possible presence of some inhibitory activity antagonizing hormonal action. Cells were incubated for 10 min at 37°C in the presence or absence of 10^{-6} M epinephrine. Incubation

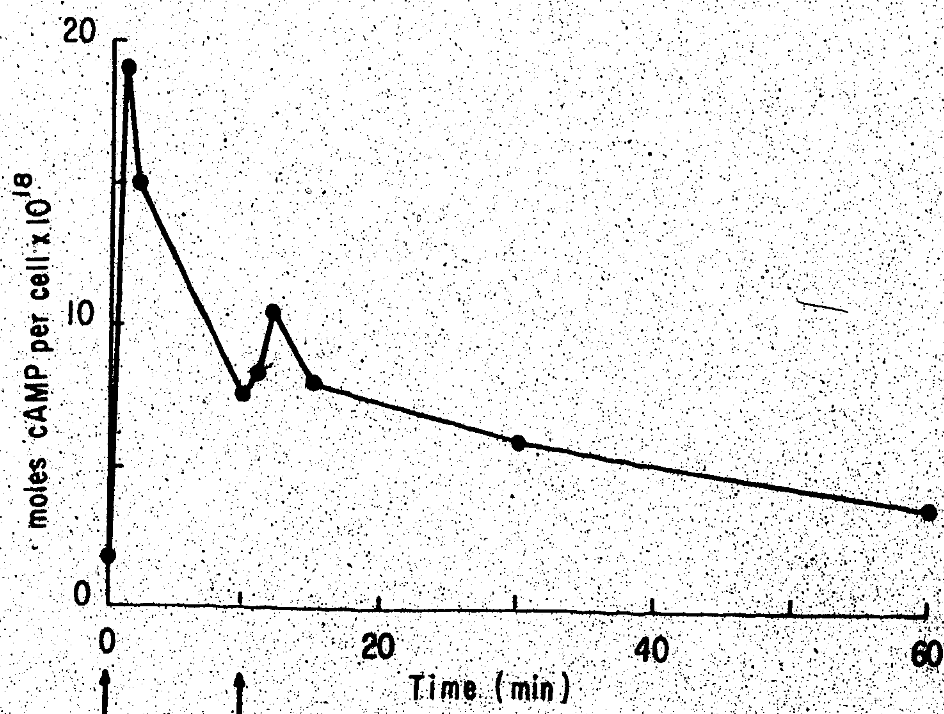
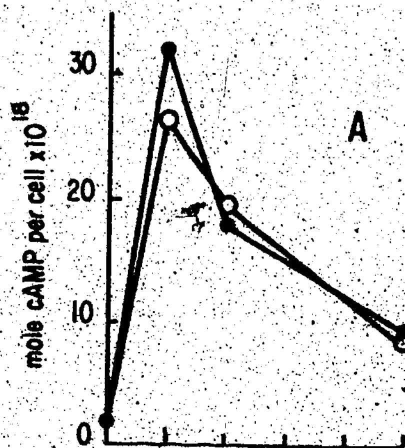
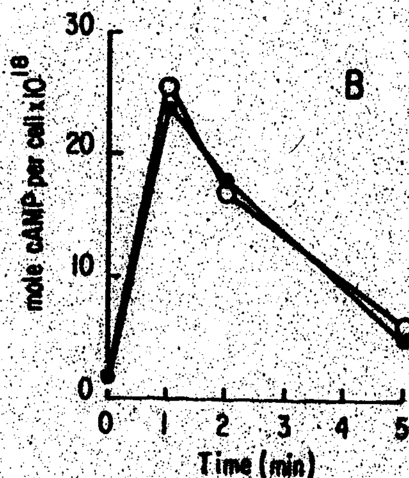


Figure 11. Effect of a second addition of epinephrine. Epinephrine (10^{-6}M) was added (0 time) to an Ehrlich cell suspension (3%, v/v) preincubated in drug free medium for 15 min. After 10 min, a second addition of epinephrine was made to the same cell suspension and the incubation continued. The level of cAMP was monitored at intervals following the first and second additions of epinephrine. Results are from a single experiment. The arrows indicate the times of the additions of epinephrine.

media from control and treated cells were then investigated for their ability to elevate cAMP levels in fresh cells. The results from two experiments are displayed in Figures 12A and 12B. It is evident that incubation media from hormone-treated suspensions did not prevent the elevation of cAMP levels in fresh cells; furthermore, the elevation of cAMP by medium from hormone-treated cell suspensions followed the same time course as observed with medium from control suspensions. Thus, no inhibitory activity was associated with the incubation medium from hormone-treated cell suspensions and the decline phase of cAMP is not due to the hormone dependent release of an antagonist into the incubation medium. These experiments provide further proof that significant breakdown of hormone does not occur during the 10 min period of the first incubation.

McKenzie and Bär (1973) demonstrated that adenosine (1 mM) significantly inhibited fluoride and epinephrine-stimulated adenylate cyclase prepared from Ehrlich cells. Furthermore, adenosine has been shown to antagonize hormone action in isolated fat cells (Schwabe *et al.*, 1973). It is known that Ehrlich cells actively metabolize adenosine following cellular uptake (Lomax and Henderson, 1973). We thus investigated the fate of exogenous adenosine spectrophotometrically under our incubation conditions (Figure 13). After 2 min in the presence of cells, extracellular adenosine was fully degraded to inosine and possibly other metabolites. The wavelength of maximum absorbance after 2 min had already shifted from 259 mμ, characteristic of adenosine, to 249 mμ, characteristic of inosine. After periods of 10 and 30 min, the maximum absorbance remained at 249 mμ but markedly decreased with time. Considering the rapidity of hydrolysis (possibly catа-





Figures 12A and 12B. Ability of medium from a hormone-treated cell suspension to elevate cAMP in fresh Ehrlich cells. To test for the possible association of some inhibitory activity with the incubation medium of hormone-treated cell suspensions, cells were incubated under standard conditions for 10 min in the presence or absence of 10^{-6}M epinephrine. Treated and untreated suspensions were then centrifuged at 3000*g* for 10 min at 4° and the supernatants collected. Epinephrine (10^{-6}M) was then added to the supernatant from the untreated suspension which was then incubated in the absence of cells for 10 min at 37°. The supernatant from hormone-treated cells was stored on ice during that time. Supernatants from untreated (closed circles) and hormone-treated (open circles) cell suspensions were subsequently investigated using standard procedures for their ability to elevate cAMP content in fresh cells. Results from two independent experiments are displayed.

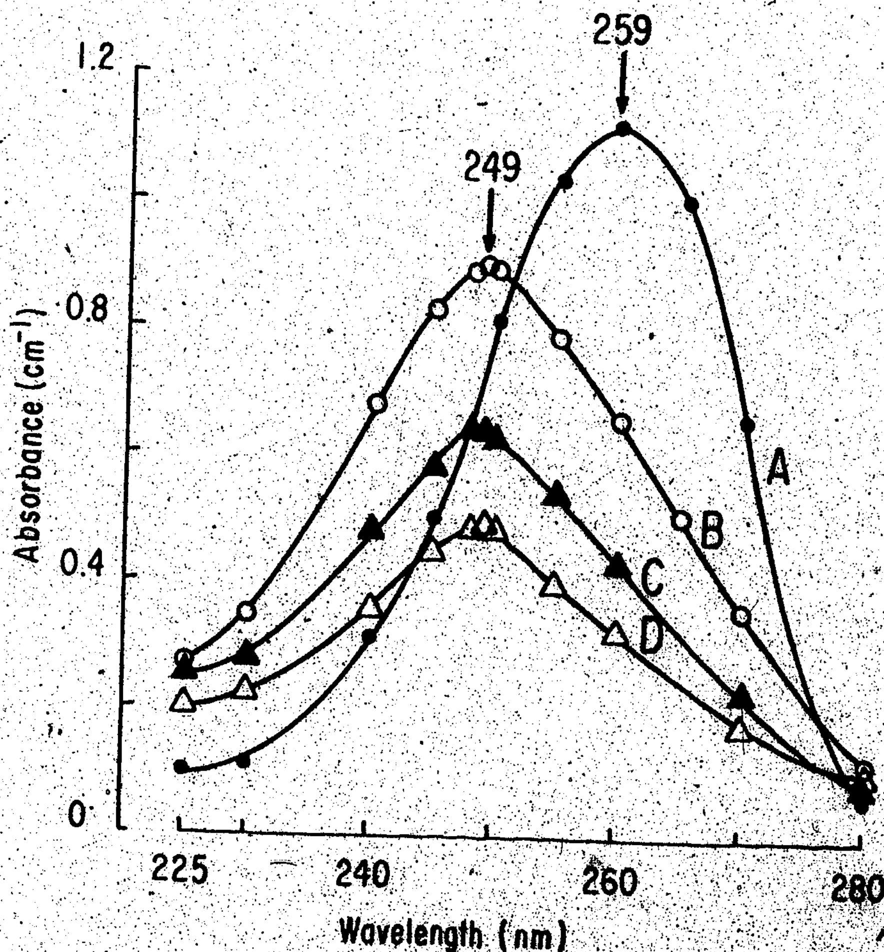


Figure 13. Fate of adenosine added to Ehrlich cell suspensions. The fate of exogenous adenosine added to an Ehrlich cell suspension was investigated spectrophotometrically as follows: adenosine was added to a 3% (v/v) Ehrlich cell suspension to obtain a final concentration of about 10^{-4}M. A control suspension did not receive adenosine. After 2, 10 and 30 min, aliquots of the cell suspensions were withdrawn, centrifuged for 0.5 min in a Superspeed Eppendorf centrifuge (16000*g*) and the absorbance spectrum of the supernatants was immediately traced between 225 and 280 nm. Corresponding aliquots of supernatants from the control suspension served as blanks for the supernatants from adenosine-treated suspensions. Spectra are displayed for supernatants prepared 2, 10 and 30 min after the addition of adenosine and are labelled B, C and D, respectively. The 0 time spectrum, labelled A, was traced for a solution of adenosine added to cell free incubation medium to obtain the same concentration as above.

lyzed by intra- and extracellular enzymes) it is unlikely that adenosine could act as an antagonist of hormone action in this system.

3.8 *Effect of propranolol added after epinephrine*

The effect of propranolol (10^{-5} M) on cAMP content in Ehrlich cells preincubated with 10^{-6} M epinephrine for 10 min was investigated. It was previously shown that 10^{-5} M propranolol completely antagonized the effect of 10^{-6} M epinephrine without affecting cAMP levels when present alone (Figure 8A). Figure 14 displays the effect of a late addition of propranolol to three cell suspensions preincubated with epinephrine for 10 min. The rate of decline of cAMP content was accelerated in the presence of propranolol compared to that in the presence of hormone alone. However, this accelerated decline was not as rapid as might have been expected from studies of the effect of propranolol on epinephrine-stimulated adenylate cyclase preparations from Ehrlich cells (Bär, 1974), where immediate and complete return to a basal rate of cAMP production was observed. This discrepency could result from different kinetics of association and dissociation of hormone and hormone antagonist to intact cells as compared to isolated membranes. Alternatively, intracellular cAMP may in fact be hydrolyzed only slowly by phosphodiesterase(s) within Ehrlich cells.

The rate of the decline of cAMP was accelerated in the presence of propranolol (Figure 14). Since propranolol alone does not affect cAMP content in Ehrlich cells, adenylate cyclase must still have been epinephrine-stimulated between 10 and 60 min after the addition of hormone.

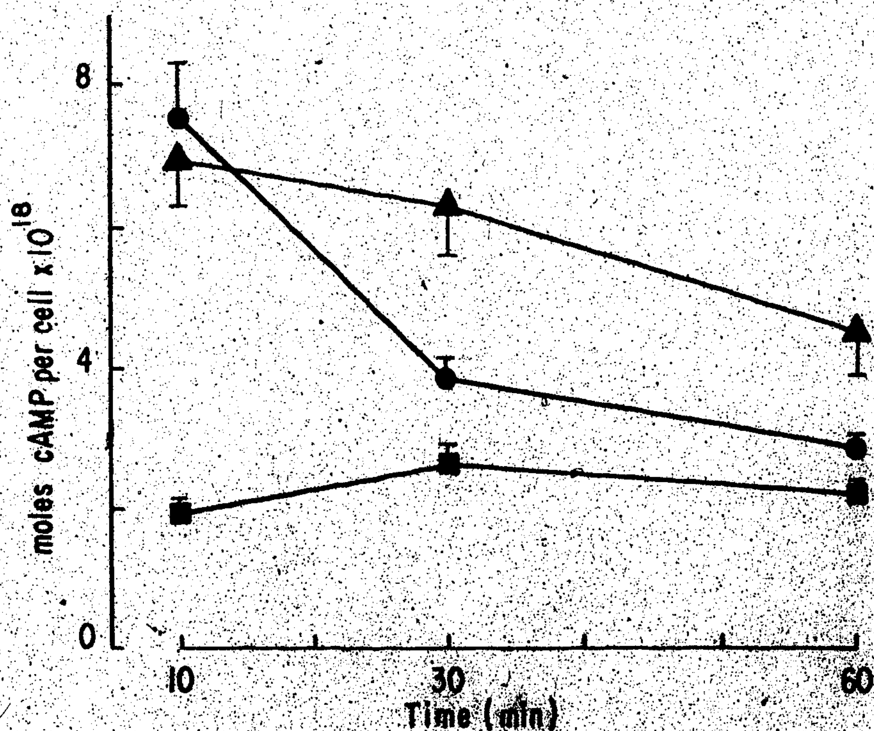


Figure 14. Effect of a late addition of propranolol. A 3% (v/v) Ehrlich cell suspension (10 ml) was incubated with 10^{-6}M epinephrine under standard conditions for 10 min; one-half of the suspension then received propranolol (10^{-6}M; closed circles) whereas the remaining half received an equal volume of saline (closed triangles). A control suspension received saline only at 0 and 10 min (closed squares). Control, epinephrine-treated and epinephrine/propranolol-treated suspensions were monitored 10, 30 and 60 min after 0 time (time of addition of hormone) for cAMP content using standard procedures. Each point is the mean from three experiments; the vertical bars represent standard errors of the means.

3.9 *Effect of theophylline added after epinephrine*

It will be remembered from Figure 5 that qualitative aspects of the time course of cAMP elevation after the addition of epinephrine remained unchanged in the presence of 1 mM theophylline. Furthermore, it was suggested that theophylline simply reduced the rate of breakdown of cAMP to the same extent throughout the entire time course for periods up to 60 min. It then became of interest to investigate the rates of rise of cAMP upon addition of theophylline (1 mM) to Ehrlich cells preincubated for various periods of time in the presence of epinephrine. Any increases in cAMP content would presumably be a measure of rates of formation of cAMP at the indicated times. Figure 15 illustrates the results from such a study. Theophylline caused an abrupt rise of cAMP content within 2 min in all instances; shorter time intervals were not investigated. It is evident that the rate of rise of cAMP in response to the addition pf theophylline decreased markedly as the time of exposure to hormone increased. Theophylline additions made 5, 10, 30 and 60 min after epinephrine caused cAMP content to elevate by 7.1, 5.0, 1.8 and 2.6 × 10^{-18} moles cAMP per cell within 2 min, respectively. Furthermore, the effect of theophylline added 5 min after epinephrine represents only a fraction of the enhancement of the peak response observed when theophylline is present prior to the addition of hormone (Figure 5).

At least two mechanisms may explain these observations:

(a) An effect similar to that observed would be expected if a phosphodiesterase less susceptible to inhibition by theophylline became increasingly responsible for cAMP degradation. We have not

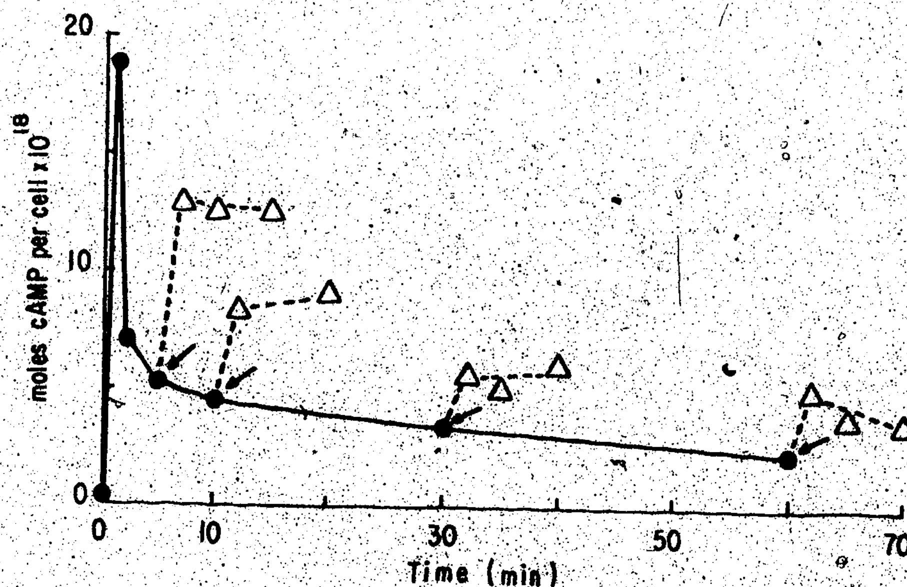


Figure 15. cAMP response of epinephrine-treated cells to late additions of theophylline. Five suspensions (3%, v/v) of Ehrlich cells extracted from the same animal were incubated under standard conditions for 15 min. Epinephrine was then added (0 time) to each suspension to obtain a final concentration of 10^{-6}M. At various intervals after the addition of hormone, theophylline (1 mM) was added to one stimulated cell suspension (theophylline additions indicated by arrows); cAMP content was monitored for periods up to 10 min after each addition of theophylline (open triangles). One suspension did not receive theophylline at any time in order to follow the time course of the cAMP response in the presence of hormone alone (closed circles).

investigated the existence of multiple phosphodiesterases in Ehrlich cells.

(b) The observed responses to theophylline would result if the rate of formation of cAMP continually decreased with time despite the continued presence of hormone.

3.10 *Partial refractoriness of stimulated cells to the effects of epinephrine*

The ability of epinephrine-pretreated and washed cells to respond to a second exposure to the hormone when resuspended in fresh medium was investigated. The results are displayed in Figure 16. Control cells incubated under standard conditions in the absence of epinephrine for periods up to 60 min were maximally responsive to epinephrine when washed and resuspended in fresh incubation medium. However, cells incubated in the presence of 10^{-6} M epinephrine for 10 min or more were partially refractory or desensitized to the effects of epinephrine when washed and resuspended in fresh medium, a treatment that restored resting cAMP levels (Figure 16). Cells incubated with 10^{-6} M epinephrine for 10, 30 and 60 min attained only 39%, 27% and 8% of control responses, respectively, at 1 min during the second exposure to epinephrine. These observations suggest that, in the presence of hormone, a time dependent increase of the ratio of cAMP degradation to formation occurs. The effects of the late additions of theophylline to epinephrine-pretreated cells (Figure 15) further support that proposal.

The dose dependence of the process which causes partial

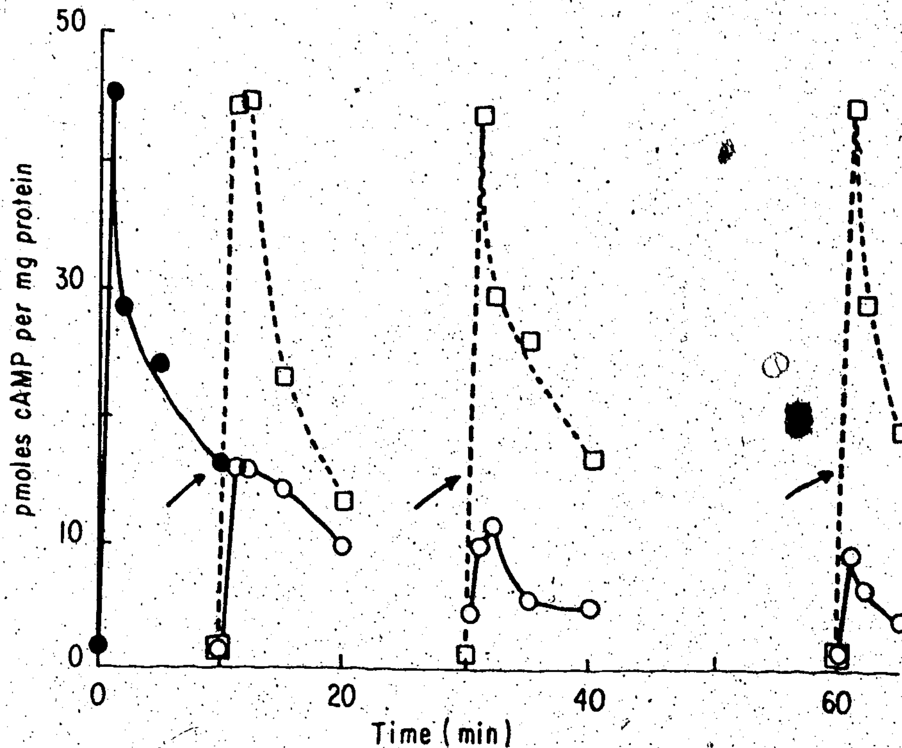


Figure 16. Ability of epinephrine-treated Ehrlich cells to respond to further additions of hormone. Ehrlich cell suspensions (3%, v/v) incubated under standard conditions in the presence of 10^{-6}M epinephrine for 10, 30 and 60 min (times indicated by arrows) were quickly diluted with 2 volumes of ice cold standard incubation medium and immediately centrifuged at 1500*g* for 5 min at 4°. The supernatants were discarded and the cells were washed in the same volume of cold medium and centrifuged as before. The washed cellular pellets were resuspended in standard incubation medium to obtain 3% (v/v) cell suspensions. The entire procedure compromising washing and resuspending was strictly set to last 20 min. Following incubation at 37° for 5 min, the recovered cells were subjected a second time to 10^{-6}M epinephrine and a time course of the cAMP response was measured (open circles; continuous lines). Control cells which were subjected to exactly the same treatment but received saline *in lieu* of epinephrine in the first incubation were similarly stimulated by 10^{-6}M epinephrine during the second incubation (open squares; broken lines). Also shown is the initial cAMP response to 10^{-6}M epinephrine during the first incubation (closed circles; continuous lines). Results are expressed as pmoles cAMP per mg acid precipitable protein.

refractoriness to hormone was investigated. Tumour cells isolated from the same animal were incubated in the presence of increasing concentrations of epinephrine and cAMP levels were measured after 1 min in an aliquot thereof to yield a dose-response curve for epinephrine action (compare with figure 10A). After 10 min in the presence of hormone, the remaining cells were centrifuged, washed once and resuspended in fresh medium; the latter treatment again restored resting cAMP levels in these cells. These cell suspensions were then incubated at 37°C for 5 min and subsequently stimulated with a dose of 10^{-6} M epinephrine; cAMP content was determined 1 min after addition of hormone. Results from one such experiment are illustrated in Figure 17. The curve marked A represents the dose-response relationship to epinephrine in the first incubation; the curve marked B indicates the extent of epinephrine sensitivity of Ehrlich cells pretreated with different doses of hormone.

The results indicate that the mechanism(s) involved in the establishment of refractoriness was activated almost maximally by an incubation for 10 min in the presence of 10^{-7} M epinephrine (curve B), a concentration which caused less than half maximal elevation of cAMP in response to hormone in the first incubation (curve A). There is no apparent correlation between the peak level of cAMP reached in the first incubation and the resulting extent of hormone desensitization. This experiment was repeated and an identical result was obtained.

The observed lack of sensitivity of epinephrine-pretreated cells to a second exposure to the same hormone is presumably due to the same phenomenon responsible for the abrupt decrease of cAMP levels in epinephrine-treated cells after 1 min (Figure 5). Since we have

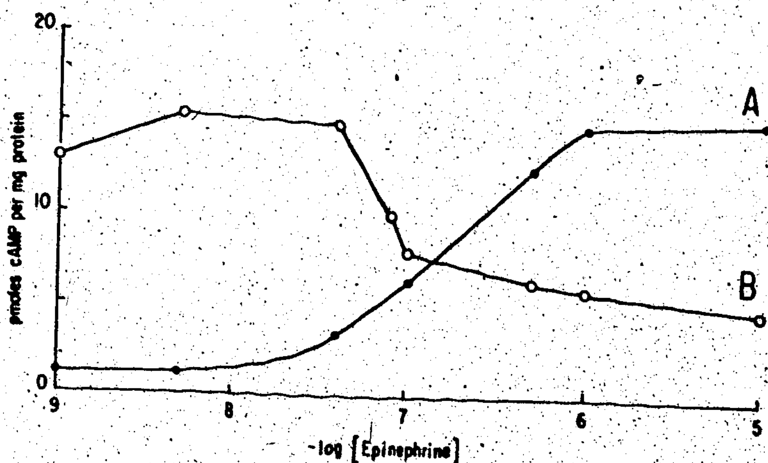


Figure 17. Dose-dependence of the desensitization of Ehrlich cells to hormonal action. Suspensions (3%, v/v) of Ehrlich cells from the same animal were preincubated under standard conditions for 15 min and then subjected to increasing doses of epinephrine; cAMP responses were measured after 1 min (curve A). After 10 min in the presence of hormone, the same cell suspensions were quickly diluted with 5 volumes of cold incubation medium and centrifuged at 1500*g* for 5 min at 4°. The supernatants were discarded and the cells were washed with the same volume of cold incubation medium. The final washed cellular pellets were resuspended in standard incubation medium to obtain 3% (v/v) cell suspensions. Each cell suspension was then incubated under standard conditions for 5 min and subsequently stimulated with a standard dose of 10^{-6}M epinephrine; cAMP content was determined after 1 min (curve B). Results are expressed as pmoles cAMP per mg acid precipitable protein. A further experiment with Ehrlich cells from a different animal yielded exactly the same result.

found no inhibitory activity in the incubation medium, this partial desensitization to epinephrine is presumably the result of an intracellular regulatory mechanism activated as a result of the hormone treatment itself. If this mechanism is activated by intracellular cAMP (an assumption in line with the findings shown in Figure 17), it is evident that it is activated almost maximally by a relatively small increase in cAMP.

A closer look at Figure 17 will reveal that although 4×10^{-8} M epinephrine caused a 2.8 fold increase of cAMP content in the first incubation, no loss of sensitivity to epinephrine was seen. Likewise, the same concentration of hormone caused a 2.2 fold increase in a repeated experiment but again no loss of sensitivity was observed. Presumably, the threshold cAMP levels necessary for activation of the mechanism leading to partial loss of sensitivity to hormone lies above a 2 to 3 fold increase over resting levels. Possibly for the same reason, pretreatment of Ehrlich cells with 1 mM theophylline (a treatment that yields a 2 to 2.5 fold increase of cAMP) does not abolish or suppress the response to hormone (Figure 5).

It appears from Figure 17 that pretreatment of Ehrlich cells with 5×10^{-9} M epinephrine, a subthreshold dose with respect to elevation of cAMP, caused a slightly enhanced response to 10^{-6} M epinephrine in the second incubation. This finding was confirmed in a repeated experiment; however, we have not investigated this effect further and it remains unexplained.

3.11 *Hormone-dependent modification of adenylate cyclase*

The possibility that epinephrine action directly involves an alteration of the adenylate cyclase enzyme system in Ehrlich ascites cells was investigated. For that purpose, basal and stimulated adenylate cyclase activity were assayed in membrane preparations from Ehrlich cells preincubated in the presence and absence of 10^{-5} M epinephrine. Three aliquots from the same tumour cell suspension were preincubated for 5 min at 37°C. The cell suspension named ET (epinephrine-treated) then received 10^{-5} M epinephrine. Suspensions labelled CA (control A) and CB (control B) received an equal volume of saline in this step. After 10 min at 37°C under standard conditions, all three cell suspensions were quickly cooled to 0°C in an iced water bath. Epinephrine (10^{-5} M) was then added to the cell suspension labelled CB and an equivalent amount of saline was added to the CA and ET cells. To summarize, ET cells were epinephrine-pretreated at 37°C; CA cells served as a control with respect to the entire manipulation while CB cells received epinephrine at 0°C, thus serving as a control to the possible carry-over of epinephrine into the subsequent adenylate cyclase preparation.

The three cell suspensions were immediately processed concomitantly as described under Methods to prepare adenylate cyclase-containing membrane preparations. Basal as well as epinephrine- and NaF-stimulated activities were immediately assayed. Results from two such experiments are reported in Table 2. It is evident that basal cyclase activity was not significantly different for membrane preparations from either ET, CA or CB cells. However, adenylate cyclase

Table 2. Effect of epinephrine-pretreatment of isolated Ehrlich cells on adenylate cyclase. The results are from two experiments using Ehrlich cells extracted from different animals. For each experiment, three 10 ml aliquots from the same Ehrlich cell suspension (3%, v/v) were incubated under standard conditions at 37° for 5 min. Cells labelled ET (epinephrine-treated) then received epinephrine (10^{-5}M) while cells labelled CA (control A) and CB (control B) received an equivalent volume of saline. After 10 min at 37°, all three suspensions were quickly cooled to 0° in iced water. Epinephrine (10^{-5}M) was then added to CB cells while an equivalent volume of saline was added to CA and ET cells. After 1 min, all cell suspensions were processed as described under Methods for the preparation of a membrane fraction containing adenylate cyclase. Results are from triplicate determinations and means ± S.E. are listed. The columns headed "% inhibition" indicate the reduction in specific activity of CB and ET cells relative to CA cells.

TABLE 2

EFFECT OF EPINEPHRINE-PRETREATMENT OF ISOLATED EHRLICH CELLS ON ADENYLATE CYCLASE

		Adenylate cyclase activity (pmoles cAMP/mg protein/min)				
Exp no.	Cells	Basal	$+10^{-4}$M Epinephrine	(% inhibition)	$+10^{-2}$M NaF	(% inhibition)
99A	CA	13.8±0.9	52.0±1.4		122.1 *	
	CB	13.4±0.5	41.2±0.7	(20.8)	108.4±3.8	(11.2)
	ET	12.8±1.0	20.7±0.7	(60.3)	103.3±5.4	(15.5)
99B	CA	11.7±0.5	66.4±1.4		109.3±3.4	
	CB	14.2±0.7	53.7±1.1	(19.1)	106.5±5.3	(2.6)
	ET	12.7±0.8	22.3±0.4	(66.5)	103.3±7.4	(5.5)

* mean of duplicate determination (121.7, 122.5)

prepared from CB and ET cells exhibited approximately 20% and 60% reduction of epinephrine (10^{-4} M) -stimulated activity, respectively, compared to cyclase prepared from CA cells. The NaF-stimulated activity was also decreased in CB and ET cyclase preparations but only to a relatively slight extent (Table 2).

The overall dilution of 10^{-6} M epinephrine during the preparation of adenylate cyclase from ET and CB cells was better than 5000 fold; thus, the effective concentration of epinephrine carried over into the adenylate cyclase assay system would be less than 2×10^{-10} M and would not be expected to produce any stimulation of adenylate cyclase. The fact that CB cells produced a cyclase preparation with reduced (20%) sensitivity to epinephrine when compared to CA cells could indicate that epinephrine can act on Ehrlich cells at 0°C and perhaps cause desensitization to the effects of epinephrine at that temperature. It has been shown by our laboratory that adenylate cyclase prepared from Ehrlich cells can be effectively stimulated by epinephrine at temperatures as low as 0°C (Bär, 1974a).

These results were essentially confirmed in two further experiments using 10^{-6} M epinephrine in the pretreatment (ET cells) step. In these experiments, hormonal sensitivity of adenylate cyclase was determined over the range 10^{-8} to 10^{-4} M epinephrine, as illustrated in Figure 18. It appears that epinephrine-pretreatment resulted in an overall reduction of the responsiveness of adenylate cyclase without a change in the maximally and half-maximally effective concentrations of epinephrine.

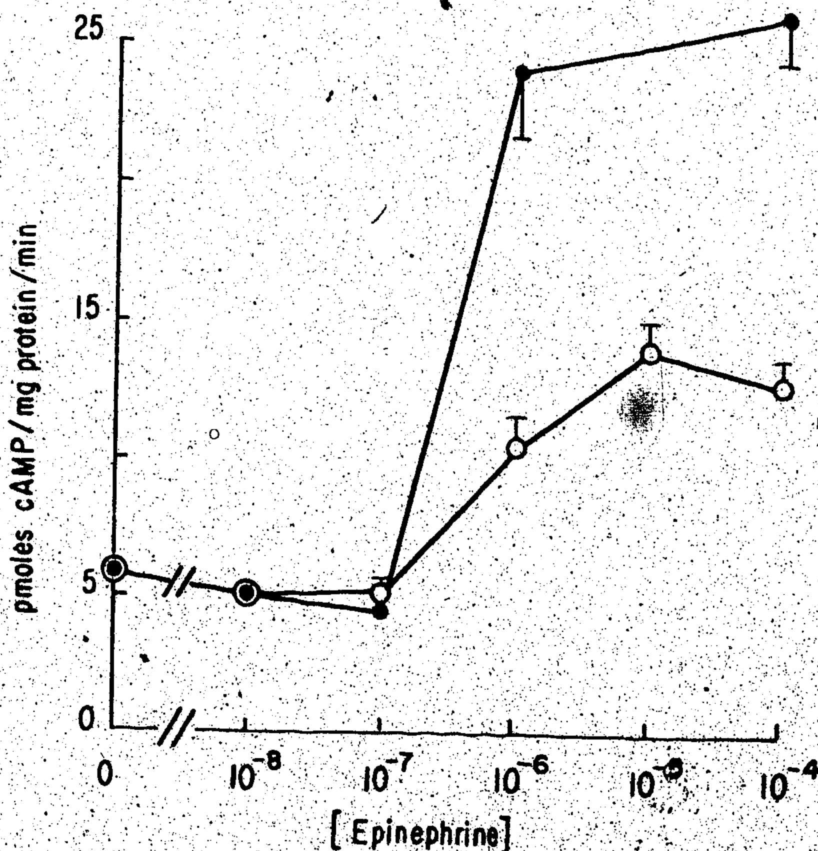


Figure 18. Response of adenylate cyclase prepared from untreated and epinephrine-pretreated Ehrlich cells to stimulation by epinephrine. Two 10 ml aliquots from the same Ehrlich cell suspension (3%, v/v) were incubated under standard conditions for 10 min. One group then received epinephrine to obtain a final concentration of 10^{-6}M; an unchallenged group received an equivalent volume of saline. Incubation was continued for a further 10 min at 37°. Membrane preparations containing adenylate cyclase were then prepared as described under Methods (section 2.8) for epinephrine-treated (open circles) and untreated (closed circles) cell suspensions and assayed in the presence of increasing doses of epinephrine. Results are from triplicate determinations and the vertical bars represent standard errors of the means when large enough to be displayed. A further experiment with Ehrlich cells from a different animal yielded essentially identical results.

3.12 *A proposed mechanism for hormone-induced desensitization of adenylate cyclase*

Although not proven conclusively by present experiments, it is most likely that the refractory behaviour of epinephrine-pretreated cells when exposed a second time to hormone, the lack of sensitivity to epinephrine of adenylate cyclase prepared from epinephrine-pretreated cells and the secondary phase of decline of cAMP levels initiated 1 min after the addition of hormone to Ehrlich cells are a consequence of a common mechanism. It is also reasonable to assume from results presented earlier (Figure 17) that an increase of cAMP content above a certain threshold level is necessary for desensitization to hormone and that this nucleotide then activates a "feedback" mechanism terminating or limiting prolonged hormone action. Since all biological effects of cAMP seem to be due to the activation of cAMP-dependent protein kinases, we propose that a cAMP-induced phosphorylation step--presumably directly on the level of hormone-sensitive adenylate cyclase--is responsible for the three related phenomena listed above.

Experimental evidence from literature supports the latter model. Constantopoulos and Najjar (1973) have postulated the existence of an inhibited "phospho" form and an active "dephospho" form of adenylate cyclase in leukocyte and platelet membranes. Phosphorylation of the enzyme could be accomplished by membrane-associated, cAMP-stimulated protein kinases since such activities appear to be present in membrane preparations from leukocytes and platelets (Najjar and Constantopoulos, 1973). Phosphoprotein phosphatases would be responsible for the dephosphorylation of adenylate cyclase and thus the

restoration of the activated form of the enzyme. Peritoneal granulocytes and blood platelets possess active, membrane-bound phosphoprotein phosphatases (Layne *et al.*, 1973). Accordingly, we suggest that the components necessary for the proposed feedback inhibition of hormone-stimulated adenylate cyclase by cAMP could be in close association as a multienzyme complex within the structure of the cell membrane.

Results from yet another membrane system provide further support for the model we propose. Membrane-associated, cAMP-stimulated protein kinases in human erythrocytes catalyze the phosphorylation of two specific protein components of the same erythrocyte membrane (Rubin and Rosen, 1973). The element of specificity evident in the latter study suggests that the phosphoesterification proposed to be responsible for the establishment of the refractory behaviour of Ehrlich cells to hormone may also be specific for some component of the cyclase system.

The proposed model is in accordance with all experimental data presented in the present thesis. The 1 min lag period preceding the phase of decline of cAMP after the addition of hormone to Ehrlich cells may represent the time necessary for the elevation of cAMP, the activation of the postulated protein kinase and the subsequent membrane alteration leading to the observed desensitization or refractoriness to hormone.

The effect of theophylline added at various times after epinephrine (Figure 15) can also be explained in the light of the present theory. At any given time, cAMP levels within Ehrlich cells are the resultant of the rates of synthesis and degradation. If we assume that theophylline causes the same degree of phosphodiesterase inhibi-

tion irrespective of the time of addition, the progressively smaller rise of cAMP in response to addition of theophylline to epinephrine-stimulated cells (Figure 15) is best explained by assuming that the cyclase system itself becomes more inhibited with time in the presence of hormone.

The present model satisfactorily explains the maintenance of partial desensitization to the effects of epinephrine when hormone-pretreated cells are washed and resuspended in fresh medium (Figure 16). The restoration of normal sensitivity of Ehrlich cells to epinephrine would be dependent on the rate of dephosphorylation of the phosphorylated membrane component. The presence of phosphatases in adenylate cyclase preparations from Ehrlich cells has been observed in this laboratory (Simonson and Bär, 1974). As yet, the time course of the restoration of normal responsiveness of adenylate cyclase and cAMP response of intact Ehrlich cells to epinephrine have not been examined.

3.13 *Hormonal stimulation of Ehrlich cells in vivo*

After having established the characteristics of the time course of the cAMP response in Ehrlich cells subjected to epinephrine *in vitro*, it became of interest to explore the effects of the same hormone on cAMP in tumour cells growing within the peritoneal cavity of mice. Table 3 summarizes the effect of i.p. administration of epinephrine (0.2 ml of 10^{-5}M solution), theophylline (0.2 ml of 23.8 mM solution) and theophylline plus epinephrine (theophylline was administered 10 min prior to the epinephrine injection) on cAMP in

TABLE 3

THE EFFECT OF EPINEPHRINE AND THEOPHYLLINE ON cAMP LEVELS IN EHRLICH CELLS *in vivo*

Treatment (concentration, volume)	Time after administration (minutes)	cAMP content (pmoles cAMP/µg DNA) means ± S.E.(*n*) ***
saline, 0.2 ml	5	0.378±0.077(3)
23.8 mM theophylline, 0.2 ml	15	0.451±0.045(2)
10^{-5}M epinephrine, 0.2 ml	5	0.671±0.066(3)
theophylline plus epinephrine *	5	1.303±0.334(3)
propranolol plus epinephrine **	5	0.238±0.029(2)

* theophylline (23.8 mM, 0.2 ml) was administered 10 min prior to epinephrine (10^{-5}M, 0.2 ml); cAMP content was monitored 5 min after the epinephrine injection.

** propranolol (10^{-4}M, 0.2 ml) was administered 3 min prior to epinephrine (10^{-5}M, 0.2 ml); cAMP content was monitored 5 min after the epinephrine injection.

*** n=number of tumours (mice) extracted for each determination.

Ehrlich cells. Likewise, the effect of propranolol (0.2 ml of 10^{-4} M solution) on the *in vivo* cAMP response of Ehrlich cells to epinephrine was investigated; the propranolol solution was administered 3 min prior to hormone. All drugs were dissolved in saline. In all instances, the cAMP content of Ehrlich cells was measured 5 min after the administration of epinephrine.

Ehrlich ascites cells subjected to the effects of epinephrine and epinephrine-theophylline combined *in vivo* responded with 1.8 and 3.5 fold increases of cAMP content above saline controls, respectively, at 5 min after the injection of hormone (Table 3). Treatment with theophylline alone for 15 min (an exposure time identical to that used for theophylline in the epinephrine-theophylline combined group) did not significantly elevate cAMP content in Ehrlich cells *in vivo*. Furthermore, propranolol totally blocked the effect of epinephrine on the cAMP response in Ehrlich cells *in vivo* (Table 3), an observation consistent with *in vitro* observations.

3.14 *Time course of the cAMP response of Ehrlich cells to epinephrine in vivo*

The time course of the elevation of cAMP in Ehrlich cells subjected to epinephrine *in vivo* was investigated. The results are summarized in Table 4. Treatment with epinephrine (0.2 ml of 5×10^{-5}M solution) resulted in 4.7 and 1.8 fold increases of cAMP above saline controls after 2 and 5 min, respectively. Combined treatment with epinephrine (same dose as above) and theophylline (0.2 ml of 23.8 mM solution administered 10 min prior to hormone injection) yielded 12.2 and

TABLE 4

TIME COURSE OF cAMP RESPONSE OF EHRLICH CELLS *in vivo* TO EPINEPHRINE AND THEOPHYLLINE

Time	cAMP content (pmoles cAMP/μg DNA; means ± S.E.(n)) after			
	saline (0.2ml)	epinephrine (5×10^{-5}M, 0.2ml)	theophylline (23.8 mM, 0.2ml)	E + T*
2 min	0.30±0.01(2)	1.40±0.44(2)		3.63±1.67(3)
5 min		0.56 (1)		1.18±0.05(3)
1 hour		0.29±0.01(2)	0.36±0.06(3)	0.17±0.05(3)
2 hours	0.28±0.05(3)			0.21±0.03(3)
4 hours			0.18±0.05(3)	0.27±0.04(3)
8 hours	0.18±0.05(3)		0.24±0.02(3)	0.17±0.01(3)

* theophylline (23.8 mM, 0.2 ml) was administered 10 min prior to epinephrine (5×10^{-5}M, 0.2 ml); the times indicated are those after the injection of epinephrine.

3.9 fold increases of cAMP at 2 and 5 min after administration of hormone, respectively. After 1 hour, cAMP levels had fallen to control values in all treated groups and these levels were maintained in all instances until at least 8 hours. Theophylline alone did not affect the cAMP content of Ehrlich cells at any time.

It is apparent that the time course of the cAMP response of Ehrlich cells to epinephrine *in vivo* follows the same pattern as that observed using isolated cells. Furthermore, theophylline enhanced the response of Ehrlich cells to epinephrine *in vivo* but did not prevent the early decline of cAMP, an observation consistent with *in vitro* investigations reported earlier (Figure 5). The *in vivo* responses are presumably influenced by redistribution and metabolism of hormone and theophylline within the animals. Hence the effects of the drugs may be terminated quite rapidly after administration and this may account for the lack of effect in all treated groups after 1 hour.

4. GENERAL DISCUSSION

As stated above, explanations other than the model proposed in section 3.12 might still account for the observed desensitization of adenylate cyclase due to treatment by hormone. In the following, some of these possibilities will be discussed as well as the biological significance of the hormone-induced desensitization of hormonal control of cAMP.

Our results do not rule out conclusively the possibility that specific hormone antagonists are formed within Ehrlich cells as a result of treatment with hormone. However, no such antagonist was found associated with the cell free incubation medium of stimulated Ehrlich cells. The possibility of an intracellular antagonist is conceivable but was not investigated. However, if such a mechanism is involved, our findings require that the inhibitory factor be tightly associated with adenylate cyclase such that desensitization persists following isolation of the enzyme.

The desensitization of intact Ehrlich cells could similarly be due to a hormone-induced enhancement or induction of *de novo* synthesis of cyclic nucleotide phosphodiesterase. The latter possibility has been demonstrated by Bourne *et al.* (1973) in cultured lymphoma cells; however, in view of the rapidity with which refractoriness to hormone is established in our system, it is unlikely that this mechanism could account for our findings. The extensive loss of catecholamine sensitivity in human diploid fibroblasts pretreated with isoprenaline, most likely a consequense of the same phenomenon operating in Ehrlich cells, was not accompanied by any

significant increase in phosphodiesterase activity (Franklin and Foster, 1973). However, in the latter study, sonic extracts of human fibroblasts were assayed for phosphodiesterase activity and such broken cell preparations cannot be regarded as true estimates of actual phosphodiesterase activity within whole cells. Subcellular organization and macromolecular associations which are disrupted by ultrasonic desintegration may be important determinants of actual phosphodiesterase activity within intact cells.

If the activity of membrane-associated phosphodiesterase was enhanced as a result of the hormonal treatment of Ehrlich cells, the effect could be carried into the membrane preparation used to assay adenylate cyclase. Hence, an enhancement of the breakdown of cAMP during the assay could be wrongly interpreted as a decreased activity of the cyclase. However, basal, epinephrine- and NaF-stimulated activities would presumably be affected similarly. Since basal and NaF-stimulated activities remained unchanged as a result of pretreatment of Ehrlich cells with hormone, it is unlikely that a phosphodiesterase effect can account for the hormone-induced desensitization of adenylate cyclase. Furthermore, the assay conditions used for adenylate cyclase are such that interference from phosphodiesterase is eliminated or minimized (Bär, 1975).

The desensitization of adenylate cyclase to hormonal stimulation could involve the loss or alteration of hormone binding sites. For example, an agonist-induced conformational change in the receptors associated with the regulatory subunit of adenylate cyclase is an attractive proposition. Following such a conformational change, the cyclase system may become susceptible to phosphorylation, refer-

ring to the model proposed in section 3.12. Such a mechanism would explain the hormone specificity of hormone-induced desensitization of adenylate cyclase observed in human diploid fibroblasts (Franklin and Foster, 1973) and guinea pig macrophages (Remold-O'Donnell, 1974). The recovery to a sensitive conformation would be necessary to allow reactivation (dephosphorylation) of adenylate cyclase. The recovery of isoprenaline sensitivity in isoprenaline-pretreated human diploid fibroblasts was found to be incomplete even after a 24 hour incubation in drug free medium (Franklin and Foster, 1973). In view of the slowness of the recovery, the authors suggest that the restoration of normal hormonal sensitivity of adenylate cyclase may require the *de novo* synthesis and assembly of functional receptors. This possibility constitutes an alternative to our suggestion that phosphatase action is involved in the recovery. The use of inhibitors of protein synthesis during the recovery phase could provide useful data to confirm of disprove either suggestion.

The occupancy of the hormone receptor site by a metabolite of the hormone remains possible. The metabolite would need to be tightly associated with the hormone receptor in order to be carried over into the membrane preparation used in the adenylate cyclase assay. By subjecting Ehrlich cells to radioactive epinephrine and subsequently analyzing the identity of labelled agents extracted from membrane preparations with organic solvents, any metabolites tightly associated with the plasma membrane could perhaps be demonstrated.

The question arises finally whether the findings presented here, and those reported in the literature, on the hormone-induced desensitization of adenylate cyclase are of physiological signific-

ce. Cells and tissues are probably subjected only rarely to high concentrations of hormone for prolonged periods of time under normal physiological conditions. However, partial desensitization of adenylate cyclase may be achieved even upon exposure to low concentrations of hormone. Tachyphylaxis and desensitization phenomena are well known in physiology and pharmacology. Little is known about the underlying biological mechanism and these are usually discussed in terms of the desensitization of the respective drug receptor systems. The present findings could be related to physiologically meaningful desensitization phenomena; however, we do not want to generalize considering the absence of any data on the desensitization of adenylate cyclase in systems such as smooth muscle.

It has been observed that the sensitivity to the catecholamines of adenylate cyclase prepared from isolated Ehrlich cells varies significantly from one batch of cells to another (Bär and Henderson, 1972). In the light of the present discussion, differing levels of circulating catecholamines within the different hosts could provide an explanation for that observation. The variable cAMP response of different batches of intact Ehrlich cells to stimulation by epinephrine found here could be similarly explained.

There are pathological and pharmacological circumstances in which tissues or cells *in vivo* are subjected to high concentrations of hormones or drugs for prolonged periods of time. Under these unusual conditions, the phenomenon of adenylate cyclase desensitization may be of special physiological significance. For example, adrenal tumours continually discharge large amounts of epinephrine into the circulation. A pharmacological/therapeutic circumstance is

that of the excessive dosing with catecholamines in asthmatic patients. In such instances, desensitization to the respective agents could involve the refractoriness (tachyphylaxis) of adenylate cyclase to further effects of the hormones.

The desensitization of adenylate cyclase could also occur in the temporal regulation of hormonally controlled events such as seasonal or circadian cycles or other rhythmic, biological events.

Both prostaglandins and catecholamines have been shown to modulate the migration of macrophages *in vitro* (Koopman *et al.*, 1973) and to accumulate at sites of infection (Willis, 1970). Furthermore, adenylate cyclase prepared from macrophages pretreated with catecholamines and prostaglandins display greatly reduced sensitivity to the effects of the same hormone used in the pretreatment(Remould-O'Donnell, 1974). Hence, both hormone and target cell seem to be concentrated in a specific anatomical area for prolonged periods of time, and hormone-induced desensitization of the cAMP response of macrophages could play a physiological role in host defense mechanisms in infection.

5. FUTURE PROJECTIONS

Whereas the experimental results presented in this thesis bear on the mechanism responsible for the secondary decline of cAMP in Ehrlich cells in the continued presence of hormone, many important and new questions are posed and remain to be challenged. Some of these are listed and discussed briefly in this section.

(a) The dependence on the dose of epinephrine of the refractory behaviour of the cAMP response of Ehrlich cells to epinephrine was reported earlier (Figure 17). It would then be of interest to determine in parallel the dose-dependence of the hormone-induced desensitization of adenylate cyclase. Both phenomena would be expected to show the same dependence on the dose of epinephrine if the refractory behaviour of the cAMP response of intact Ehrlich cells to epinephrine is a consequence of the desensitization of the adenylate cyclase system to that hormone.

(b) We have not yet investigated the effect of pretreatment of Ehrlich cells with prostaglandin E_1 on the hormonal sensitivity of subsequently prepared adenylate cyclase. It will be of interest to see whether adenylate cyclase desensitized to the stimulatory effect of epineprhine is also insensitive to prostaglandin E_1 and *vice versa* or whether the alteration of the cyclase is specific for the effect of each hormone. On the basis of reports by others (Remold-O'Donnell, 1974; Franklin and Foster, 1973), the latter is expected to be true.

(c) Present findings have shown that cAMP is elevated in Ehrlich cells *in vivo* following administration of epinephrine alone

or in combination with theophylline. Furthermore, the early decline of cAMP levels following the addition of hormone *in vitro* was observed *in vivo*. Hence, it would be of interest to demonstrate whether adenylate cyclase prepared from Ehrlich cells pretreated with epinephrine *in vivo* displays partial desensitization to the effects of the same hormone under assay conditions *in vitro*.

(d) In order to demonstrate whether cAMP itself is involved in the mediation of the desensitization phenomenon, intact Ehrlich cells could be incubated *in vitro* in the presence of high concentrations of cAMP or its derivatives. The effect of this treatment on the hormone sensitivity of adenylate cyclase could then be examined. It must be remembered that a negative answer would not preclude a role of cAMP in desensitization. In particular, the presence of hormone bound to the regulatory subunit of adenylate cyclase may be a requirement for desensitization to occur.

(e) The model we have proposed in section 3.12 involves the cAMP-induced phosphorylation of a membrane component of Ehrlich cells. Hence, Ehrlich cells equilibrated with ^{32}P-inorganic phosphate and subsequently stimulated with hormone would be expected to incorporate ^{32}P into a specific component of the cell membrane at an accelerated rate during the first few minutes after the addition of hormone. Demonstration of the specificity of such a phosphoesterification, if indeed it takes place, would be of special interest.

(f) The dephosphorylation and thus reactivation of adenylate cyclase under the proposed model (section 3.12) would involve phosphoprotein phosphatases. The study of intra- as well as extra-

cellular phosphatases would thus be of interest.

(g) It would also be of general interest to investigate cyclic nucleotide phosphodiesterases in Ehrlich cells and to establish directly whether these enzymes can play a role in hormone-dependent desensitization of the cAMP response of intact Ehrlich cells. Our arguments to the contrary are based on indirect evidence only.

(h) Finally, it would be of interest to investigate whether the same model applies to other cell and tissue systems to determine how widespread and thus how significant hormone-induced desensitization of the cAMP response to hormone is *in vivo*.

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APPENDIX A

As pointed out in the Introduction, the ultimate goal of the present study was to provide a rational approach to investigations attempting to correlate increased intracellular cAMP content to a reduced rate of growth of Ehrlich ascites tumours in mice. In the context of the present work, it has not been possible to explore this problem in any depth. However, a few relevant experiments have been performed and, in spite of their limited character, they are described here since we feel that the findings may provide direction for future developments.

Two main approaches were adopted in these investigations. The first consisted of the daily administration of cAMP alone or in combination with theophylline into the peritoneal cavity of tumour bearing mice. In a second approach, repeated administrations of epinephrine alone or in combination with theophylline were investigated with respect to their effect on the rate of growth of Ehrlich ascites tumours.

Two methods were used to assess cellular material present in the Ehrlich ascites tumours. Total tumour cell number was determined using a Coulter electronic particle counter after proper dilution of cells extracted from the peritoneal cavity of mice. Packed cell volume (ml) was also utilized to assess cellular material present in the ascites tumours (Patt and Straube, 1956).

Table 5 summarizes the effect of the intraperitoneal administration once daily of 0.2 ml sterile solutions of saline, 12.5 mM cAMP (0.82 mg daily), 23.8 mM theophylline (2 mg daily) and cAMP and

TABLE 5

EFFECT OF cAMP AND THEOPHYLLINE TREATMENTS ON THE SIZE OF EHRLICH ASCITES TUMOURS

Group number	Treatment (concentration, volume)	Tumour cells per mouse × 10^{-7} means ±S.E. (n)
1	saline, 0.2 ml	15.3±1.89 (12)
2	12.5 mM cAMP, 0.2 ml	13.6±2.65 (14)
3	23.8 mM theophylline, 0.2 ml	15.8±3.13 (11)
4	theophylline+cAMP*	9.60±2.15 (12)

* cAMP (12.5 mM) *and theophylline* (23.8 mM) *were combined in the same injection in a total volume of* 0.2 ml.

Table 5. Drugs dissolved in saline to the indicated concentrations were administered i.p. to tumour bearing mice according to the following schedule: 24 hours after implantation of 2.5 million Ehrlich cells into the peritoneal cavity of albino ICR mice (4 to 6 weeks of age, 20 to 25 grams) treatment was begun and carried out at 24 hour intervals for a total of six injections. The total number of cells per ascites tumour was determined 24 hours after the last injection using a Coulter electronic particle counter.

theophylline combined in the same injection. All drugs were dissolved in saline. The experiment was scheduled as follows: 24 hours after implantation of 2.5×10^6 Ehrlich cells into the peritoneal cavity of randomly bred ICR mice (4 to 6 weeks of age, 20 to 25 g), treatment was begun and carried out at 24 hour intervals for a total of 6 injections. The total number of tumour cells present within the peritoneal cavity of all mice was determined 24 hours following the last injection using a Coulter counter after proper dilution of the extracted cells in saline.

It is evident from Table 5 that tumour-bearing mice which were subjected to the combined treatment of cAMP and theophylline bore fewer cells than did mice treated with saline, cAMP alone or theophylline alone. The mice subjected to the combined treatment bore only 61% of the mean number of tumour cells present in mice which received saline only. However, a one-way analysis of variances indicated that the mean cell numbers obtained were not significantly different for any one group of mice with respect to any other group at the 5% level of significance. Therefore, the observed effect of the combined treatment was only marginal and its meaning remains equivocal.

In an experiment similar to the one reported here, Seller and Benson (1973) reported a 50% reduction of Ehrlich ascites tumour growth in response to the administration twice daily of cAMP plus theophylline. The effect of the combined treatment was reported to be significant at the 1% level of significance. The total daily doses of cAMP and theophylline for 20 g mice were 0.4 mg and 2 mg, respectively. In our experiment reported above, corresponding doses for cAMP and theophylline were 0.82 mg and 2 mg, respectively. Thus,

daily doses of theophylline did not differ but we administered two times the dose of cAMP. Two other differences exist between our experimental design and that of Seller and Benson. They implanted 10 million tumour cells whereas we implanted only 2.5 million cells. Furthermore, they initiated treatment 72 hours after tumour implantation and maintained injections twice daily for 4 to 5 days; we began treatment 24 hours after implantation and maintained injections once daily for 6 days. The strains of Ehrlich cells used were probably different, too, and that of Seller and Benson could conceivably be more responsive to increased cAMP than the one employed in the present study.

In a second series of experiments, we investigated the effect of the administration twice daily of 0.2 ml sterile solutions of saline, epinephrine (5×10^{-5} M), theophylline (23.8 mM) and epinephrine plus theophylline on the rate of growth of Ehrlich ascites tumours. The treatment schedule was designed as follows: 24 hours after implantation of 2.5 million tumour cells into the peritoneal cavity of healthy, 4 to 6 week old ICR mice (20 to 25 g), treatment was begun and repeated every 12 hours until four injections had been made. The mice were then left undisturbed for 36 hours after which time cellular material within the peritoneal cavity of all mice was determined. Packed cell volume (ml) was used to assess cellular material present in the ascites tumours. The results are summarized in Table 6.

It is evident from Table 6 that mice subjected to the combined treatment of theophylline and epinephrine bore fewer tumour cells than did mice administered saline, epinephrine alone or theo-

TABLE 6

EFFECT OF EPINEPHRINE AND THEOPHYLLINE ON THE SIZE OF EHRLICH ASCITES TUMOURS

Group number	Treatment (concentration, volume)	Packed cell volume (ml) ± S.E. (n)
1	saline, 0.2 ml	1.09±0.15 (13)
2	5×10^{-5}M epinephrine, 0.2 ml	1.11±0.13 (12)
3	23.8 mM theophylline, 0.2 ml	0.98±0.19 (12)
4	theophylline+epinephrine*	0.66±0.16 (15)

* theophylline (23.8 mM, 0.2 ml) was administered 10 min prior to epinephrine (5×10^{-5}M, 0.2ml)

Table 6. Drugs dissolved in saline to the indicated concentrations were administered i.p. to tumour bearing mice according to the schedule described in the text. After six days, total cellular material present within the peritoneal cavity of the mice was extracted, centrifuged at 1500*g* for 5 min in a clinical centrifuge and packed cell volume was determined. Results are expressed as means ± S.E.

phylline alone. The mice subjected to the combined treatment bore only 60% of the mean number of cells present in the saline-treated group. Epinephrine or theophylline alone had no effect on tumour size expressed in the form of pooled data (means ± S.E.).

We have plotted the group distributions of the packed tumour cell volume per mouse for each group (Figure 19). Class intervals of 0.4 ml were chosen. Figure 19 plots the midpoint of each class against the fraction of the total number of mice from each group bearing packed cell volumes corresponding to the respective classes. Just as the mean packed cell volume for the saline- and epinephrine-treated groups were very similar (Table 6), their group distributions were likewise very similar. The theophylline-treated group, on the other hand, displayed a group distribution that suggests the existence of two different populations of tumour cells. We suggest that the smaller packed cell volumes result from host-tumour combinations specifically affected by the theophylline treatment while the second population exhibiting larger packed cell volumes reflects the existence of host-tumour combinations that display an unaltered or slightly increased tumour size as a result of theophylline treatment. A similar bimodal distribution was observed for the theophylline-treated group from the experiment reported in Table 5. In the present experiment, results expressed in the form of pooled data show that the mean packed cell volume for the theophylline-treated group varied only slightly from that of the saline- and epinephrine-treated groups (Table 6).

The group distribution for the epinephrine/theophylline-treated group displayed a pronounced shift toward very small packed

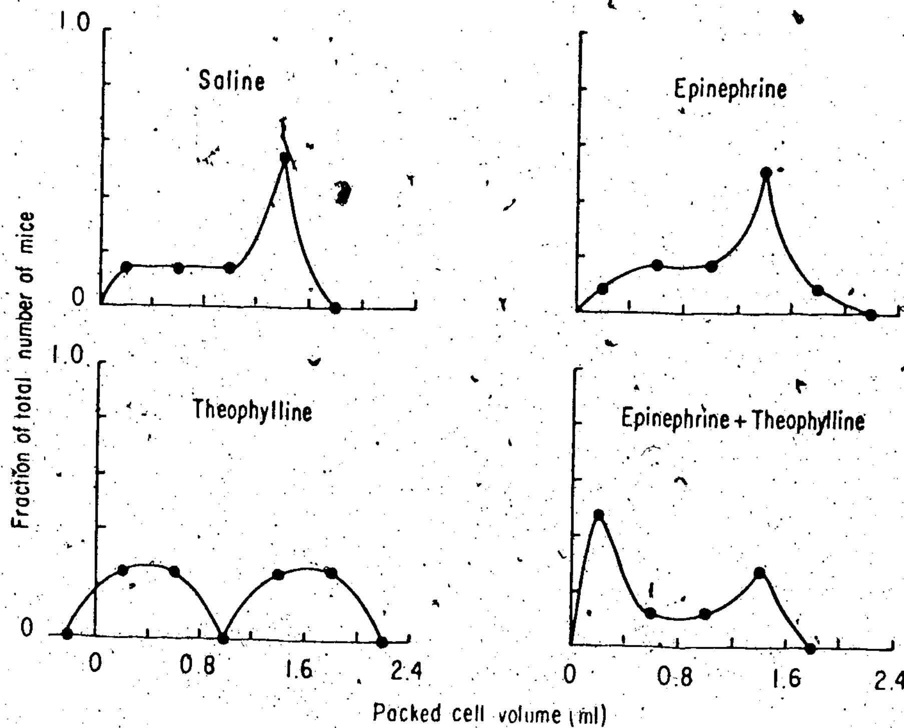

Figure 19. Group distribution for the packed tumour cell volume (ml) per mouse for each group from Table 6. Class intervals of 0.4 ml were chosen; the midpoint of each class (abscissa) is plotted against the fraction of the total number of mice from each group (ordinate) bearing cellular volumes corresponding to the respective classes.

cell volumes (0 to 0.4 ml). However, tumour size in some mice remained unaffected by a treatment with epinephrine plus theophylline. Similarly, some tumours subjected to the effects of cAMP plus theophylline remained unaffected by that treatment although other tumours displayed reduced size (observations from the group distributions for the experiment described in Table 5). Three main conclusions are suggested by these findings:

(a) theophylline appears to be the active agent mediating the decreased tumour size observed in some treated animals.

(b) the combination of cAMP or epinephrine with theophylline appears to enhance the effect of the latter on tumour size; cAMP or epinephrine alone have no effect at the doses studied.

(c) the effect of theophylline alone or in combination with cAMP or epinephrine on tumour size appears to involve the host and thus may not be a direct carcinostatic action on the tumour cells.

Furthermore, epinephrine administered in a dose which causes a moderate but transient increase of cAMP content had no effect on the parameters determining tumour size. Possibly, the increase in cAMP following administration of epinephrine alone is too small or transient to mediate an effect on cellular growth. However, when theophylline is included, the duration and magnitude of the cAMP response to epinephrine are enhanced and that could explain the observed effect of the combined treatment. However, the variable response of different tumours to the combined treatment can hardly be explained without proposing an effect at the level of the host.

Our findings do not deny nor suggest that cAMP plays a role in the regulation of Ehrlich ascites tumour growth. However, since the

effect of the combined treatments of epinephrine or cAMP with theophylline seem to be host-dependent, a mechanism involving an effect of the administered agents on the immunological response of the genetically different hosts is possible and must be borne in mind.

In the future, these experiments should be repeated. Although it may appear more rational to inject hormones at more frequent intervals, such an approach may be of limited applicability due to the following reasons:

(a) the obvious technical problems involved in subjecting mice to repeated injections at short intervals for prolonged treatment periods.

(b) the presently reported desensitization of the cAMP response of Ehrlich cells to repeated exposures to epinephrine.

We have considered the use of catecholamines covalently bound to minute glass beads; such preparations have been reported to retain their biological activity (Venter *et al.*, 1972). However, it is not clear at present whether the biological activity of such preparations is actually due to hormone bound to the glass matrix or to pharmacological concentrations of catecholamines leaching off the glass beads (Yong, 1973).

Preliminary experiments have indicated that epinephrine covalently attached to reactive glass beads elevates cAMP content in Ehrlich cells *in vitro* according to the same time course as free epinephrine. Furthermore, preliminary toxicity studies have indicated that large amounts of the catecholamine glass (.2 g) can be implanted within the peritoneal cavity of mice without evidence of severe toxic effects for periods up to 1 week. However, the minute glass particles

have clumped and become encapsulated by the host's defense mechanisms within 48 hours of their implantation. Hence, this approach is also of limited applicability and the use of "slow-release" forms of the catecholamines now seems to be the most logical approach for future experimentation.