CHANGES IN TESTIS CYCLIC NUCLEOTIDE METABOLISM

DURING TROUT SPERMATOGENESIS

by

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ABSTRACT

The concentrations of cyclic GMP and cyclic AMP, in rainbow trout (Salmo gairdnerii) testis, were determined during hormonally-induced spermatogenesis. In immature trout testis, cyclic GMP and cyclic AMP concentrations were approximately equal ($\approx 2\mu$mol/kg wet weight). An abrupt 10 fold decrease in cyclic GMP occurred during mitotic activity and testis growth prior to meiosis. Cyclic AMP decreased 2 fold during this time. A further gradual 5 fold decrease in cyclic GMP occurred during the remainder of spermatogenesis. No significant change in cyclic AMP occurred during development after the initial 2 fold decrease.

Cyclic AMP and cyclic GMP phosphodiesterase activities, measured at both high (millimolar) and low (micromolar) substrate concentrations were determined in trout testis during hormonally-induced spermatogenesis. When measured at millimolar substrate, cyclic GMP phosphodiesterase activities did not change significantly throughout spermatogenesis. Cyclic AMP phosphodiesterase activities, measured at millimolar substrate, decreased about 50% prior to meiosis and then increased, during spermatid differentiation, to attain a final activity slightly greater than that observed in immature testis. When measured at micromolar substrate, in the presence of EGTA, cyclic GMP phosphodiesterase activities

decreased about 50%, while cyclic AMP phosphodiesterase
activities, measured under the same conditions, increased
20 fold during the course of spermatogenesis.   A detailed
study of cyclic GMP phosphodiesterase activities, measured
at micromolar substrate, in the presence and absence of Ca^{2+},
showed there was no change in Ca^{2+}-dependent cyclic GMP
phosphodiesterase activity at the time of development at
which the large decrease in cyclic GMP is observed.

DEAE-cellulose profiles of cyclic nucleotide phosphodi-
esterase activities, from trout testis at different stages
of development, showed two peaks of cyclic AMP activity and
one peak of cyclic GMP activity.   The latter cochromato-
graphed with the first cyclic AMP activity peak.   A large
increase in the first cyclic AMP phosphodiesterase peak
occurred when spermatids were maturing, without a concurrent
increase in cyclic GMP phosphodiesterase activity.   This
indicates the induction of a specific, high-affinity cyclic
AMP phosphodiesterase during the meiotic stage of testicular
development.

Phosphodiesterase assays using micromolar substrate
concentrations, on subcellular fractions, demonstrated that
about 85% of cyclic AMP hydrolysis and 80% of cyclic GMP
hydrolysis was soluble.   Both peaks of cyclic AMP activity
were observed in the DEAE-cellulose profile of a 100,000xg
supernatant (soluble) fraction from trout testis.   The
small amount of particulate cyclic AMP phosphodiesterase
activity, in the 100,000xg pellet fraction, was associated

mainly with the second peak on the DEAE-cellulose profile.

Kinetic analyses of homogenate phosphodiesterases from mature testis showed only high affinity cyclic AMP activity (apparent Kms for cyclic AMP of 1.1 µM and 0.3 µM) and both low and high affinity cyclic GMP activity (apparent Kms for cyclic GMP of 220 µM and 8 µM). Kinetic analyses of cyclic AMP hydrolysis by the two peaks of phosphodiesterase activities purified on DEAE-cellulose, confirmed the presence of high affinity activities.

Guanylate cyclase activites were assayed in the soluble and particulate fractions from immature testis and from testis at different stages of hormonally-induced development. There was an approximate 1:2 ratio of soluble to particulate guanylate cyclase activities in immature and in mature trout testis. A 3 fold decrease in both soluble and particulate guanylate cyclase activities coincided with the 10 fold decrease in cyclic GMP concentration observed in maturing trout testis. Thus, in trout testis during spermatogenesis, cyclic GMP concentrations reflect the developmental modulation of guanylate cyclase activity, rather than that of cyclic GMP phosphodiesterase.

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DEDICATION

This thesis is dedicated to my Granny whose immense love and wisdom have so greatly enriched my life.

ABBREVIATIONS

cyclic AMP	adenosine 3',5'-monophosphate
cyclic GMP	guanosine 3',5'-monophosphate
AMP, GMP, CMP, UMP	the 5'-monophosphates of the ribonucleosides of adenine, guanine, cytosine and uracil
ATP, GTP	the 5'-triphosphates of the ribonucleosides of adenine and guanine
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
pmol, μmol	picomoles (10^{-12}) , micromoles (10^{-6})
M	molar (moles per liter)
μM, mM	micromolar, millimolar
EGTA	ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetate
EDTA	ethylenediaminetetraacetate
TCA	trichloroacetic acid
DEAE-cellulose	diethylaminoethyl cellulose
PEI-cellulose	polyethyleneimine cellulose
Tris	tris(hydroxymethyl)aminomethane
MIX	1-methyl-3-isobutyl xanthine
POPOP	1,4-di[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyl oxazole
SCINTREX	tradename for a universal liquid scintillation cocktail of 1:1 Triton X-100: Toluene, containing POPOP and PPO
TTP	a scintillation fluid consisting of Toluene:Triton X-100:PPO, 60:125:1.32 v/v/w
A_{254}	amount of material with an optical density of one in a one cm pathlength
xg	times the force of gravity

Ci	Curie
rpm	revolutions per minute
cpm	counts per minute
°	degrees Centigrade
V	velocity
S	substrate
Km	Michaelis Menton constant; the substrate concentration at which half maximal velocity of an enzyme activity occurs
app Km	apparent Km; the substrate concentration at which half maximal velocity of an enzyme activity occurs, when there are multiple Kms for the same activity with differing enzyme concentrations
AG	analytical grade ion exchange resin prepared by sizing and purifying the standard DOWEX resin of the same designation
AG 1-X8	quaternary ammonium anion exchange resin; medium pore size

INTRODUCTION

A. Spermatogenesis

Spermatogenesis is a complex process of sequential developmental steps during which immature, diploid germ cells differentiate into highly specialized haploid spermatozoa (1). In the vertebrate testis, there is an ordered sequence of cytological and biochemical changes, which have been studied extensively (1). However, many details of the regulation of spermatogenesis remain unresolved. These include the control of DNA replication, transcription and translation, the induction of cellular differentiation, and the precise nature of hormonal control by the pituitary gonadotropins and the testicular androgens. Since the course of spermatogenesis, in terms of specific timing of DNA, RNA and protein synthesis, is known (1) possible roles for regulatory molecules, such as the cyclic nucleotides, in the control of growth and differentiation of testicular cells, can be investigated.

There is evidence for the involvement of cyclic AMP during mammalian spermatogenesis, as an intracellular mediator of the pituitary gonadotropin hormones, follicle-stimulating (FSH), and luteinizing hormone (LH) (2, 3, 4). FSH and LH stimulate the production of cyclic AMP in their respective target cells, the Sertoli cells (3) and the Leydig cells (4). In FSH-stimulated Sertoli cells, the cyclic AMP produced by adenylate cyclase has been shown to activate a cyclic AMP-dependent protein kinase and to result in de novo synthesis of a new protein, the androgen binding protein (5). In

LH-stimulated Leydig cells, cyclic AMP has been shown to
enhance the production of the major testicular androgen,
testosterone (6).

A precise role for cyclic GMP has not yet been defined
in any system.   In several cell lines, cyclic GMP has been
found to enhance mitosis (7), and DNA (8) and RNA (9) synthesis.
Since these activities are characteristic of cells in the early
stages of spermatogenesis, a role for cyclic GMP in such cells
is possible and requires further investigation.

The cytological and biochemical changes observed in germ
cells during spermatogenesis are basically similar for all
vertebrates (1).   The different cell types are characterized
by their size, time of appearance during testis development,
the appearance of their cytoplasmic and nuclear contents,
and their relative rates of DNA, RNA and protein synthesis (1).
The infantile testis contains gonocytes which have arisen
from primordial germ cells.   At a specific time in development
the gonocytes multiply and are transformed into spermatogonia.
Spermatogonia continue to multiply by a series of mitotic cell
divisions, the number of which varies from species to species.
In mammals there are 4 to 6 divisions, while in fish there are
about 10 to 12 divisions (1, 10).   Cells deriving from one
parental spermatogonial cell remain partially cojoined through-
out differentiation by cytoplasmic bridges (11).   The cells
deriving from one parental cell synchronously differentiate
into primary spermatocytes (1).   The primary spermatocytes

double their nuclear DNA to give a tetraploid (4n) content,
and then have a long prophase period during which there is
pairing of chromosomes and possible exchange of genetic
material. The primary spermatocyte finally undergoes cell
division to give two secondary spermatocytes, each with a
diploid (2n) complement of DNA. The secondary spermatocytes
are shortlived and each divides without replication of DNA
to give rise to two spermatids with a haploid (n) content of
DNA (1). This reductive division is termed meiosis. The
spermatids so formed are non-dividing and only low levels of
DNA synthesis can be detected (12). During the following
stages of spermatid differentiation, the volume of the
spermatid is greatly reduced by cytoplasmic loss and the
nuclear DNA is condensed (10). RNA synthesis ceases in
spermatids (13) while certain proteins are synthesized during
spermatid maturation (1, 14, 15). In the final different-
iation step of transformation of spermatid into spermatozoa,
new structural and enzymatic proteins are synthesized to form
the propulsive tail and the complex headpiece characteristic
of fully mature sperm (1). (Figure 2A; p13, sequence summary.)

In vertebrates with different modes of sperm production,
there are considerable differences in testicular structures (1).
In fish, where there is a relatively short and intense breed-
ing period, during which enormous quantities of sperm are
released, a cystic mode of sperm production is used (16).
The testes of salmonids, to which family the rainbow trout,

Salmo gairdnerii, belongs, are made up of elongated, branching

tubular structures, known as lobules, within fibrous walls (17).

The lobules contain within them 2 or more cysts of germ cells

surrounded by connective tissues(17).   When the testes are

immature, the cysts are essentially empty of germ cells except

for a small number of gonocytes or resting spermatogonia (18).

As testicular maturation begins, the gonocytes differentiate

into spermatogonia.   In fish with several breeding seasons,

the germ cells which begin the second and following reproduct-

ive cycles are resting spermatogonia (18).   These are germ

cells which remain undeveloped during the preceeding cycle.

The spermatogonia divide rapidly filling each cyst with a

large number of germ cells.   These spermatogonia then differ-

entiate via the sequence previously outlined to produce mature

sperm.   Within each cyst, all the germ cells develop in

synchrony, but within a testis the various cysts may be at

different stages of development (19).

In the mammal, male germ cells are contained within the

seminiferous tubules in a concentric progression of maturity

from periphery to the central lumen, into which mature sperm

are continuously released after puberty (1).   Except for

in pre-pubertal development, the entire length of the seminifer-

ous tubule is continually populated with germ cells at various

stages of development (1).

In both mammals and fishes, the male germ cells are

surrounded by a framework of fibrous connective tissue that

supports the blood vessels, lymphatics and nerves of the testis (1). The distinctive component of this connective tissue is the epitheloid cell which secretes testicular androgen. These are termed Leydig cells in the mammalian testis (1) and lobule-boundary cells or Leydig cell homologues in the fish testis (19).

One other important somatic cell, the Sertoli cell, has been identified in most vertebrate testes (19), but is not easily identified in all fishes (10). In the mammal, Sertoli cells line the periphery of the seminiferous tubule (1). The Sertoli cell precursors are very sensitive to the endocrine state of the animal and in the rat continue to divide up to 20 days after birth (20). The Sertoli cell is necessary for the early stages of germ cell maturation (1). It produces an androgen binding protein (21) after FSH-stimulation and may provide nutrient support to developing germ cells (22). Sertoli cells also have considerable phagocytic activity and reabsorb residual bodies left in place after the release of sperm (22). When Sertoli cells are observed in fishes, they are found associated with structural elements of the cyst walls (19).

In all vertebrates, both the development of the germ cells and the activity of the Leydig and Sertoli cells are under the control of the anterior part of the pituitary gland, the adenohypophysis (1). In mammals, removal of the pituitary (hypophysectomy) leads to the atrophy of maturing

germ cells, degeneration of the seminiferous epithelium,
and loss of secondary sex characteristics (23).   In fish,
different species show a variety of degrees of degeneration
after hypophysectomy (16).   In salmonids, the transformation
of spermatogonia into primary spermatocytes is blocked after
hypophysectomy (24).

In mammals, the pituitary is stimulated by a hypothalamic
peptide releasing hormone to produce the glycoprotein gonado-
tropins, FSH and LH (25), and the presence of both FSH and
LH is required for the maintenance of spermatogenesis (1).
The target cell and action of each of these hormones has been
mentioned earlier in this Introduction.   In fish, the process
of spermatogenesis is also controlled by the pituitary gland
(17).   However, purification studies of pituitary extracts
from both carp (26) and salmon (27) have not revealed the
presence of more than one gonadotropin responsible for testis
development in fish.

The major external factor regulating sexual maturation
in fish is the photoperiod (28).   In salmonids, the annual
period of spermatogenic activity begins in late spring and
takes place over about 6 months, alternating with a period
of involution and reconstitution of testicular tissue during
the winter months in preparation for the next differentiation
period (18).   In trout, appropriate manipulation of the
number of hours of daylight per day can be used to accelerate
spermatogenesis (28).

Another method of acceleration of the natural rate of
spermatogenesis, in the trout testis, is by administering
a series of injections of salmon pituitary gonadotropin
extracts (29, 30).   It has been shown that such a series of
injections into sexually immature male rainbow trout, kept
in water of the appropriate temperature, causes the growth
and differentiation of the immature testes (29, 30).   This
results in a 500-1000 fold increase in testes wet weight.
The induced spermatogenesis biochemically and morphologically
parallels that observed in naturally maturing testis, but
testis growth occurs at twice the rate (31).   This is
probably due to the larger proportion of cysts induced to
differentiate by the artificially high dosage of gonadotropin.
The induced trout testis differentiation can be initiated at
any time of the year, providing a convenient system for the
study of biochemical changes during spermatogenesis.

In artificial hormonally-induced spermatogenesis in
trout, the testes weight doubling time is about 1 week.
This results in a logarithmic increase in testes weight for
about 8 to 10 weeks until the final stages of testicular
development (4 to 5 weeks).   In these final stages of spermat-
ogenesis, a logarithmic decrease in wet weight occurs, due
to germ cell cytoplasmic loss and tissue degeneration (18).

After 2 weeks of salmon pituitary extract injections,
the predominant germ cell type is the spermatogonium. A
typical picture of the cell types present in trout testis at
this stage is shown in Figure 1A.   Connective tissue separates

FIGURE 1

A. Upper left.   Light micrograph of a lobule from a trout
   testis after 2 weeks of salmon pituitary extract injections.
   Several cysts of spermatogonia, with spherical nuclei
   containing multiple nucleoli, are shown.   Cysts are
   separated by connective tissues.   x1,000

B. Upper right.   A view of trout testis after 6 weeks of
   hormone injections shows primary spermatocytes (center
   field) and spermatogonia (far right).   x1,250

C. Lower left.   After 8 weeks of hormone injections, trout
   testis contains primary spermatocytes (lower left) and
   smaller secondary spermatocytes (lower right).   A group
   of synchronous cells undergoing the meiotic reduction
   division can be seen in the upper portion of the light
   micrograph.   x1,250

D. Lower right.   Trout testis after 12 weeks of hormone
   injections shows small, compact spermatids and some
   large resting spermatogonia.   The breakdown of connective
   tissues between cysts is apparent.   X1,250
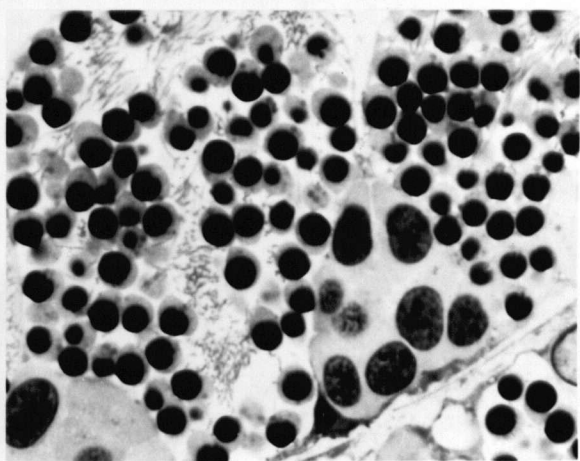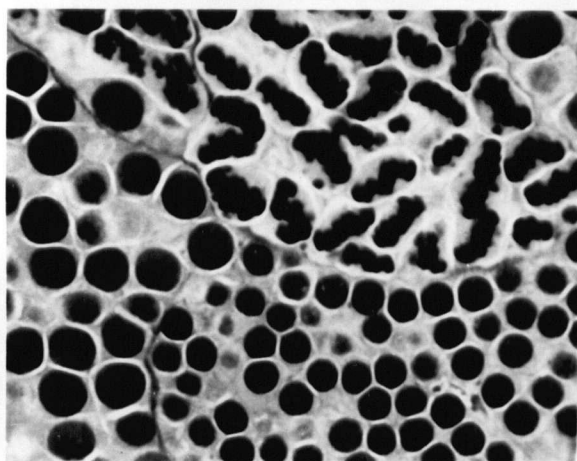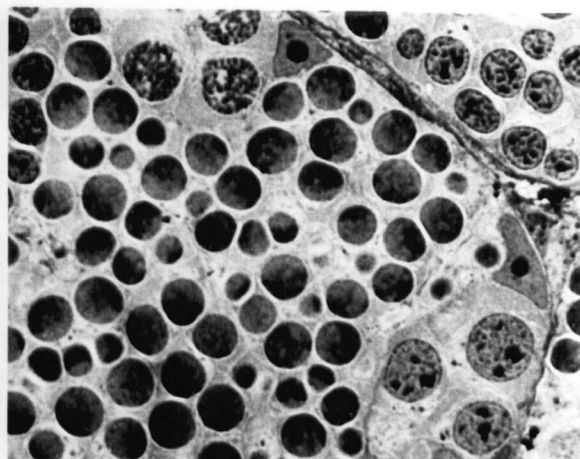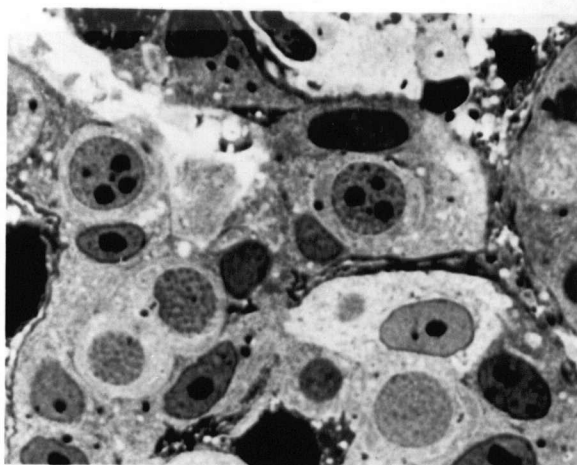
the cysts of spermatogonia. It has been estimated that spermatogonia make up about 10-15% of the total wet weight of the immature testis (10). The other 85-90% is presumed to be due to connective tissue and lymphatic and blood cells. Spermatogonia are active in DNA replication (32) and cell division resulting in rapid testis growth. Differentiation into primary spermatocytes takes place during weeks 4 to 6 of hormonal induction (32). Cells in the trout testis, at this stage of development, can be seen in Figure 1B. The primary spermatocytes differentiate into secondary spermato-cytes, which in turn produce early spermatids, from weeks 6 to 8 of hormonal induction (Figure 1C). During weeks 8 to 10 spermatids differentiate. At this stage, the replacement of histones by protamine has been studied in detail (33, 34, 35). After 10 to 12 weeks of hormone injections, mature spermatids and sperm are the predominant germ cell types in trout testis and degeneration of testis tissue occurs, as seen in Figure 1D. The presence of spermatogonia at this late stage of development reflect cells which could initiate the next spermatogenic cycle (18). In salmon, it has been shown that spermatozoa produced from a similar 10 to 12 week series of injections of pituitary gonadotropins into immature salmon, were as effective in fertilizing eggs as those from naturally maturing fish (36).

Induced spermatogenesis in trout testis is a particularly good system in which to study developmental changes in germ cells, since at any one given time there is a predominance

of one germ cell type. Previous developmental studies have
concentrated on spermatid differentiation and the replacement
on chromatin of histones by protamines (33, 34, 35). After
about 7 to 8 weeks of hormone injections, protamines are first
observed in trout testis (37). They are phosphorylated
soon after their synthesis and then bind to chromatin to cause
its initial condensation (33). A protamine kinase, which is
stimulated 1.5 -2 fold by cyclic AMP, has been partially
purified from trout testis at this stage of development (38).
Condensation of chromatin proceeds by a sequential release of
histones, which may involve histone acetylation (34). The
final transformation into sperm head nucleoprotamine is closely
linked to protamine dephosphorylation (35).

Developmental studies during spermatogenesis have also
been made in rat testis (14, 15). Analysis of specific
proteins during development has shown the formation of specific
gene products is associated with the appearance of specific
cell types (15). This is illustrated in the developmental
patterns of enzymes shown in Figure 2B. During spermatogenesis
in rat testis, characteristic sequential changes in the rates
of synthesis and phosphorylation of specific nuclear acidic
proteins have also been observed (15). It has been suggested
that it is these proteins which control the selective activ-
ation or repression of genes during spermatogenesis (15).

The cyclic nucleotides, cyclic AMP and cyclic GMP,
modulate cellular metabolism by stimulating the phosphorylation

FIGURE 2

A. Sequential cell types and their characteristics
    during spermatogenesis

B. Testicular enzyme activities related to specific
    cell types during spermatogenesis in the rat (from
    reference 15)

A.   Sequential cell types and their characteristics
                during spermatogenesis

Cell Type	Characteristics
Gonocytes ↓	2n content of DNA Little cell proliferation
Spermatogonia ↓	2n content of DNA Rapid cell proliferation Active DNA, RNA and protein synthesis

Spermatocytes
  a) Primary
  b) Secondary ↓
	4n content of DNA
	2n content of DNA; no DNA replication. RNA synthesis decreases

Spermatids ↓	n content of DNA DNA and RNA synthesis ceases Histones replaced by protamines Flagellum forms
Spermatozoa	n content of DNA Sperm specific enzymes appear

B.   Testicular enzyme activities related to specific cell
            types during spermatogenesis in the rat
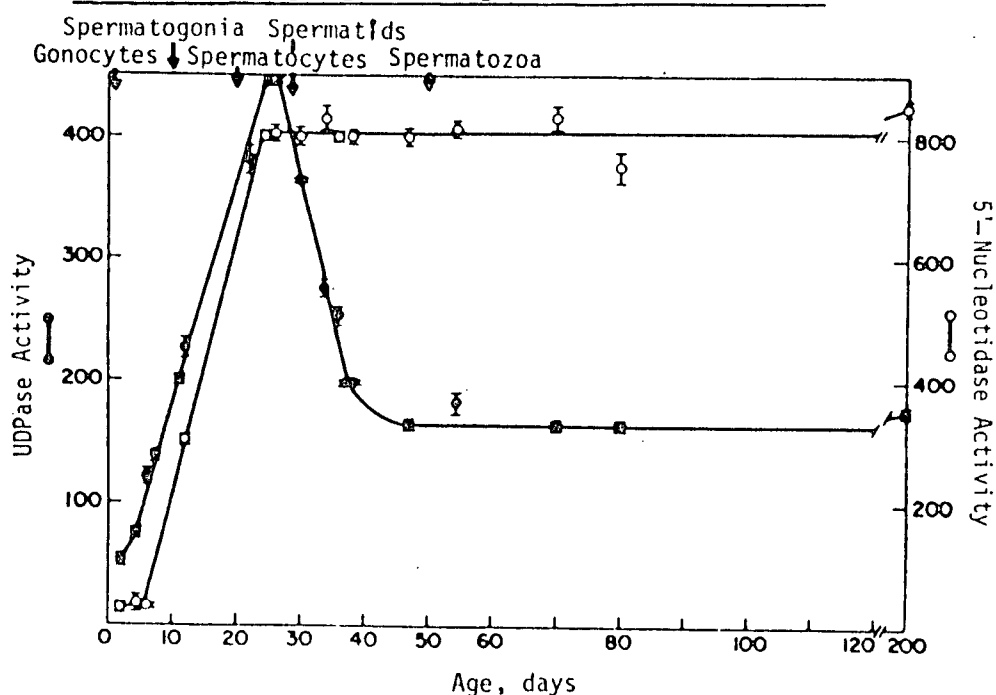
of proteins (39). Due to the importance of phosphorylation of nuclear proteins in the control of genetic expression during spermatogenesis, the role of cyclic AMP and cyclic GMP may be crucial for male germ cell differentiation.

B. The role of cyclic AMP and cyclic GMP in metabolism

Since the discovery of cyclic AMP, in 1956, by Sutherland and his associates, as the low molecular weight, heat-stable factor which mediated the intracellular effect of epinephrine and glucagon on rat liver (40), an extensive literature has been amassed indicating the importance of cyclic AMP as a cellular metabolic regulator in many tissues and organisms throughout the animal kingdom. In the initial papers from Sutherland's group, cyclic AMP was shown to be degraded by an enzyme, cyclic nucleotide phosphodiesterase, which formed adenosine 5'-monophosphate as the product of hydrolysis (41). The enzymatic conversion of ATP to cyclic AMP, by adenylate cyclase, was defined in a later series of papers from the same group, in 1962 (42, 43). They showed that the enzyme required ATP and Mg^{2+}, was particulate, was stimulated by NaF, and, in a tissue-specific manner, was stimulated by different hormones.

A mechanism by which a change in the cellular concentration of cyclic AMP could alter the metabolic state was determined by Krebs and his associates, in 1968, during an investigation on the regulation of glycogenolysis in skeletal muscle (44). Cyclic AMP was found to activate a cyclic AMP-dependent protein

kinase by binding to the regulatory subunit of the regulatory-catalytic holoenzyme causing dissociation of the regulatory subunit from the catalytic subunit which was then fully active (44). Cyclic AMP-dependent protein kinases in other tissues have the same method of activation (45, 46). Many reviews on cyclic AMP metabolism exist (47, 48, 49) and the topic will not be further discussed except with regard to spermatogenesis.

Following the discovery of cyclic AMP and its important role in cellular metabolic regulation, evidence was sought for the existence of other naturally occurring cyclic nucleo-tides (50, 51). Cyclic GMP was isolated from rat urine, in 1963 (52) and has since been found in all phyla of the animal and plant kingdom (53, 54) In eucaryotes, intracellular concentrations of cyclic GMP have been found to be elevated in response to acetylcholine (55), prostaglandin-F2α (56), fibroblast growth factor (57), and several lectins (7). However, direct stimulation of guanylate cyclases by any of these agents has not been convincingly demonstrated (54). Guanylate cyclases (58, 59), cyclic GMP specific phospho-diesterases (60, 61), and cyclic GMP-dependent protein kinases (62, 63) have also been identified and studied.

Guanylate cyclases differ from adenylate cyclases in their substrate requirement for GTP, their divalent cation preference for Mn^{2+}, their frequent equal distribution between soluble and particulate fractions, and, their insensitivity to polypeptide hormones (54). Membrane-

bound guanylate cyclases have been shown to be activated by Triton X-100 (64), by specific lipids (65, 66) and by Ca^{2+} (67). Soluble guanylate cyclases can be activated by free radical forming agents, such as nitrosamines (68), and nucleophiles, such as azide, nitrite and hydroxylamine (69). Several soluble guanylate cyclases can be activated spontaneously by air oxidation (70). Activation of guanylate cyclases by prostaglandins (and their metabolic endoperoxide products) has been suggested to be related to their oxidative ability, as well as their lipid character (54).

Cyclic GMP phosphodiesterase activities specific for cyclic GMP have been reported, while there are other phosphodiesterases which can hydrolyze both cyclic GMP and cyclic AMP with varying affinities for each cyclic nucleotide (60, 71). In a number of mammalian tissues, micromolar concentrations of cyclic GMP were shown to stimulate 2-3 fold the rate of phosphodiesterase-catalyzed hydrolysis of micromolar concentrations of cyclic AMP (72). It has been shown that Ca^{2+}, in the presence of a specific calcium-dependent phosphodiesterase protein activator (73) increases cyclic GMP hydrolysis more than cyclic AMP hydrolysis (74). Since this calcium-dependent regulator protein is in excess of phosphodiesterases in most tissues (75), it has been suggested that the regulation of cyclic GMP hydrolysis is dependent on the intracellular calcium concentration (54).

Cyclic GMP-dependent protein kinases have been shown to be activated by a somewhat similar mechanism to cyclic AMP-

dependent protein kinases, although in the latter case, each holoenzyme consists of two regulatory and two catalytic sub-units, while in the former case, the holoenzyme has only one of each subunit (76).   In many tissues, a protein modulator has been observed, which enhances cyclic GMP activation of cyclic GMP-dependent protein kinases and inhibits cyclic AMP activation of cyclic AMP-dependent protein kinases (77). Several excellent reviews on cyclic GMP metabolism are available (53, 54) and this topic will not be covered further except in relation to spermatogenesis.

Cyclic CMP and cytidylate cyclases have also been recently identified in some mammalian tissues (78), but very little is known about this cyclic nucleotide's role in biological systems.

C. The role of cyclic AMP and cyclic GMP in spermatogenesis

A study of intracellular concentrations of cyclic AMP and cyclic GMP during spermatogenesis in the rat testis (79) showed that both cyclic nucleotide concentrations were elevated in infantile testis (cyclic AMP 20 pmol/mg protein; cyclic GMP 0.6 pmol/mg protein) and both decreased at about the time of the onset of the meiotic reduction division in germ cells (20-25 days after birth).   Cyclic AMP continued to decrease until the time of the appearance of spermatids (about 35 days after birth) and then increased steadily in maturing testis to a final adult concentration of about half the initial concentration.   Cyclic GMP continued to decrease

throughout maturation, to a concentration about one-sixth of the initial concentration. This study also utilized immuno-fluorescent probes for cyclic AMP and cyclic GMP. This technique is assumed to localize cyclic nucleotides associated with cyclic nucleotide receptor proteins in the tissue because any free cyclic nucleotide should be lost during the treatment process of the unfixed frozen tissues (80). During the infantile phase of rat testicular development, fluorescence within seminiferous tubules was intense, for both cyclic nucleotides, and nuclear staining patterns of cells close to the tubular wall i.e. Sertoli cells and spermatogonia, were observed for both cyclic nucleotides. This tubular wall staining was observed throughout maturation (79). In another study, of cyclic AMP immunofluorescent localization in developing rat testis (81), the cyclic AMP tubular staining pattern was shown to persist one month following hypophysectomy in adult rats, despite undetectable plasma concentrations of FSH and LH. In the investigation in which both cyclic nucleotides were localized, cyclic AMP fluorescence was not detected in germ cells in later stages of testicular development, i.e. meiosis and spermatid differentiation, while cyclic GMP was observed associated with germ cell types during most stages of development (79). In particular, cyclic GMP was found associated with nuclear elements, specifically with the chromosomes of prophase spermatocytes during meiosis. Cyclic AMP was not observed on chromosomes at any stage of development. These results suggested nuclear roles for both

cyclic AMP and cyclic GMP in infantile development, and also, a specific nuclear role for cyclic GMP at meiosis.

Developmental studies of the adenylate and guanylate cyclases, in rat testis, have indicated some correlation of their specific activities with the observed changes in cyclic nucleotide concentrations (82, 83). Steiner and his associates showed a correlation between testis tissue cyclic GMP concentration and soluble guanylate cyclase activity during rat testicular differentiation (82). These workers concluded that elevated cyclic GMP concentrations in the infantile rat testis reflect, in part, the activity of an inducible soluble guanylate cyclase (82). Braun and his associates have also identified a soluble guanylate cyclase activity in rat testis, which was most active in premeiotic stages of development (83). This guanylate cyclase was localized to a non-germ cell tubular component of the immature rat testis (83). In rat testis depleted of germ cells by X-irradiation before birth, two specific membrane-bound adenylate cyclases have been identified (84). One is sensitive to FSH and is present in the tubular Sertoli cells and perhaps in spermatogonial cells and the other is sensitive to LH and is present in the Leydig cells (85).

A distinctive germ cell-associated soluble adenylate cyclase has been detected in the seminiferous tubules of rats, at the time of the appearance of spermatid cells (83). The specific activity of the enzyme increased about 2 fold during the period of spermatid development into mature sperm and reached maximal values in the testis of adult rats.

The timing of the appearance of this soluble adenylate cyclase in rat testis correlates with the observed increases in cyclic AMP concentrations (79). This enzyme was found to be insensitive to Mg^{2+}, fluoride, FSH and LH, and the stimulatory effect of Mn^{2+} could be potentiated by Ca^{2+} (85). In epididymal sperm, the Mn^{2+}-sensitive adenylate cyclase was found to be associated with "mitochondrial" and "microsomal" particulate fractions (83). It was suggested that this Mn^{2+}-sensitive adenylate cyclase may play a role in the transformation of spermatid cells into spermatozoa, perhaps in the formation of the sperm tail (83). In support of this statement is the previous observation that "cyclic AMP is absolutely necessary for flagella formation and hence motility, in cyclic AMP-deficient mutants of Escherichia coli and Salmonella typhimurium" (87).

Sperm of both invertebrates and vertebrates have been shown to contain equal amounts of Mg^{2+}- or Mn^{2+}-dependent particulate adenylate cyclase, but particulate Mn^{2+}-dependent guanylate cyclase was several hundred fold more active in invertebrate sperm than in vertebrate sperm (88). The extremely active guanylate cyclase in sea urchin sperm was found to be localized primarily in the flagellar plasma membrane (89). Sperm from several species of fish (salmon and herring) contained appreciable amounts of adenylate cyclase but no detectable guanylate cyclase (88).

Developmental studies on cyclic nucleotide phosphodiesterase activities during spermatogenesis are scarce. This is

probably due to the complexity of multiple, interconvertible phosphodiesterase activities which are observed in most tissues (90). One investigation of total cyclic AMP phosphodiesterase activities during rat spermatogenesis showed a 5 fold increase in activities from day 20 to day 50 after birth (91). Cyclic AMP phosphodiesterase activities then remained constant through to full maturity (80 days after birth). In this study, day 20 after birth was the first time sampled, so infantile testis activities were not determined.

Studies using the phosphodiesterase inhibitors caffeine, theophylline and papaverine showed an increase in respiration and mobility of bovine spermatozoa, an effect also produced by cyclic GMP and dibutyryl cyclic AMP (92). These studies suggested the presence of an active cyclic nucleotide phosphodiesterase in mammalian sperm. A highly specific testicular cyclic AMP phosphodiesterase was found associated with sexual maturation in the rat and rabbit (93), and in the ram (94). In the rat, one form of cyclic AMP phosphodiesterase, with a Km of 6.5×10^{-5} M was identified in immature testis and a second, high affinity enzyme, with a Km of 2.5×10^{-6} M, appeared in coincidence with the appearance of mature sperm (91). An active cyclic AMP phosphodiesterase was found in fish sperm (salmon and herring) but very low cyclic GMP hydrolysis was detected (88).

The overall balance of intracellular cyclic nucleotide concentrations may be regulated by two factors other than their

synthesis by the cyclases and their degradation by the
phosphodiesterases.   These factors are extracellular excret-
ion (95, 96) and intracellular binding to inactivatable cyclic
nucleotide binding proteins (97).

The possible importance of the role of excretion of
cyclic nucleotides in the regulation of intracellular cyclic
nucleotide concentrations, in testis cells, is unknown.   The
concentration of cyclic nucleotide binding proteins in testis
has been investigated from the point of view of studying
cyclic AMP-dependent protein kinase activity during spermato-
genesis (98, 99).   It has been suggested, on the basis of
studies on the regulation of bovine heart protein kinases (97)
that, a dephosphorylated form of holoenzyme could be resistant
to cyclic AMP-induced dissociation and could serve as a cyclic
nucleotide sink, rendering cyclic AMP unavailable for protein
kinase activation, as well as resistant to hydrolysis by cyclic
AMP phosphodiesterases.   Recently, it has been demonstrated,
using cyclic AMP-dependent protein kinases separated by DEAE-
cellulose chromatography (Type I kinase eluted by 0.1 M NaCl
and Type II kinase eluted by about 0.2 M NaCl) from 5 rat and
2 bovine tissues, that autophosphorylation of the regulatory
subunit by the catalytic subunit occurred with all Type II
enzymes but not with Type I enzymes (100).   A negative influ-
ence of Type II cyclic AMP-dependent protein kinases, on
lymphocyte mitogenesis, has been observed (101).   In devel-
opmental studies of cyclic AMP-dependent protein kinase
activities, in rat testis, Type I kinase remained constant
from birth throughout differentiation, while Type II kinase

increased from birth onwards to obtain an adult profile at

the time of the first reductive divisions (98). It is

possible that Type II cyclic AMP-dependent protein kinases

may be important in the control of effective intracellular

cyclic AMP in male germ cells during and after meiosis.

In another investigation on the changes in protein kinase

activities in rat testis during spermatogenesis, total kinase

activities (including both cyclic AMP-dependent and cyclic

AMP-independent protein kinases) decreased at about 10-20

days after birth and then a sharp increase occurred around

35-45 days of age (99). This increase correlated with an

increase in the cyclic AMP-dependent versus cyclic AMP-

independent protein kinase ratio, and also with the timing

of biochemical events such as, the increase in intracellular

cyclic AMP concentrations (79), increases in plasma and test-

icular testosterone (102), the increase in Mn^{2+}-sensitive

soluble adenylate cyclase (83), and also the increase in the

high affinity cyclic AMP phosphodiesterase activity (91). It

was suggested that all the testicular enzyme activity increases

were related to the appearance of spermatids (99).

While very active cyclic AMP-dependent protein kinases

have been observed in sperm of several mammalian species,

cyclic GMP-dependent protein kinases have not been found (88).

No developmental studies of cyclic GMP-dependent protein kinase

activities or of cyclic GMP-binding proteins, in testis from

any vertebrate or invertebrate, have been reported.

D. Objectives of this thesis

The objectives of this thesis were to determine cyclic GMP and cyclic AMP concentrations in the testis of the rainbow trout Salmo gairdnerii during spermatogenesis and to investigate the developmental modulation of the enzymes regulating these concentrations. Particular emphasis was placed on cyclic GMP metabolism, due to the lack of information on this cyclic nucleotide's role in growth and differentiation. As previously discussed, artificial hormonally-induced spermatogenesis in trout is a well-defined system, in which the stages of germ cell maturation take place as an ordered sequence of events throughout the maturing testis. Consequently, changes in the biochemistry of the tissue can be followed chronologically and correlated with distinct stages of germ cell growth and differentiation.

MATERIALS AND METHODS

A. <u>MATERIALS</u>

All radiochemicals were from New England Nuclear. [^{3}H]adenosine 3',5'-monophosphate, ammonium salt (specific activity 39.8 Ci per mmol) and [^{3}H]guanosine 3',5'-mono-phosphate, ammonium salt (specific activity 8.8 or 10 Ci per mmol) were purified by chromatography on AG 1-X8 resin, as described in Methods.

[^{3}H]guanosine (specific activity 19 Ci per mmol) was purified by paper chromatography, as described in Methods. [^{14}C]adenosine 5'-monophosphate, diammonium salt (specific activity 0.422 Ci per mmol) was converted to [^{14}C]adenosine as described in Methods.

Cyclic AMP, cyclic GMP, ATP, GTP, adenosine, guanosine, phosphocreatine, creatine phosphokinase (rabbit muscle, Type I) and MIX were all purchased from Sigma Chemical Co. Cyclic nucleotide phosphodiesterase (beef heart) was bought from Calbiochem and made up to 5 mg/ml in H_2O and stored frozen at -20° in small aliquots.

<u>E. coli</u> alkaline phosphatase was purchased from PL Biochem-icals and was made up as a 0.37 mg/ml solution in H_2O and stored frozen at -20° in small aliquots.

Bovine serum albumin, Fraction V, Lot 400427, was bought from Calbiochem.

All other chemicals were reagent grade.

Triton X-100 (tradename of Rohm and Haas for iso-octyl-phenoxy-polyethoxyethanol) was supplied by J.T. Baker Co. SCINTREX (tradename for a universal liquid scintillation cocktail of 1:1 Triton X-100:Toluene, containing PPO and POPOP) was purchased from J.T. Baker Co.

Cyclic GMP radioimmunoassay kits were purchased from Collaborative Research. The cyclic AMP kit was bought from New England Nuclear.

B. METHODS

1. Chromatography materials preparation

DEAE-cellulose (Whatman DE 32, microgranular) was washed with 0.5 N NaOH (10 volumes), distilled H_2O (20 volumes), 1 M sodium acetate, pH 6.5 (10 volumes), and then equilibrated with column buffer, i.e. 20 mM sodium acetate, pH 6.5 containing 4 mM 2-mercaptoethanol.

BioRad AG 1-X8, 200-400 mesh, chloride form, resin was prepared as described previously (103). For cyclic GMP sample purification, the AG 1-X8 resin was then poured into columns (0.7 x 3.5 cm) and prewashed with 5 N formic acid (15 ml), followed by distilled H_2O (15 ml). For the cyclic AMP sample purifications, the AG 1-X8 resin was prewashed with 5 N formic acid (10 volumes) followed by distilled H_2O, in a large glass-sintered funnel.

For cyclic GMP phosphodiesterase assays, the AG 1-X8 resin was prepared as described previously (103) and was resuspended in distilled H_2O (1:2 v/v resin to H_2O) after

addition of 1 N formic acid to a pH of 2.5. For cyclic
AMP phosphodiesterase assays, the AG 1-X8 resin was resusp-
ended in distilled H_2O (1:2 v/v resin to H_2O) after addition
of glacial acetic acid to a pH of 3.7.

2. Paper chromatography

All paper chromatography was on Whatman 40 paper using
the descending system at 23°. The solvent used was iso-
propanol:NH_3:H_2O (7:1:2 v/v/v). Nucleotides and nucleosides
were located by shortwave ultraviolet absorbance using a
hand-held Mineralight from Ultraviolet Products, California.
Ultraviolet absorbing regions which contained radioactive
compounds were cut out and eluted by the method of Heppel
(104). The eluate (0.5 ml) was added to 5 ml of TTP
scintillation fluid and the radioactivity determined in a
liquid scintillation spectrometer.

3. Preparation of [^{14}C]adenosine from [^{14}C]AMP

[^{14}C]AMP (specific activity 0.422 Ci per mmol),
100 µl (0.0018 mg) was incubated with E. coli alkaline
phosphatase (0.05 mg/ml) and 8 mM Tris.HCl, pH 7.8, in a
total volume of 130 µl, at 30° for 135 min. This mixture
was cochromatographed on paper with adenosine (1 A_{254} unit)
and AMP (1 A_{254} unit) and developed as described in the
previous section. Using these conditions there was 100%
conversion of [^{14}C]AMP to [^{14}C]adenosine.

4. Fish husbandry

Immature rainbow trout Salmo gairdnerii, 17-20 cm in length, from the Sun Valley Trout Farm in Mission, British Columbia, were kept in 200 liter, self-cleaning, fibreglass aquaria. The recirculating water was aerated and kept at 9-12° and a 13 hour light and 11 hour dark cycle was used. The fish were fed three times a week, on Oregon Moist Pellets, from the Moore-Clark Co., La Conor, Washington.

Immature male trout were selected by laparotomy and were injected intraperitoneally, twice a week, with 0.1 ml of a crude salmon pituitary extract. Injections were continued for 12 weeks, during which time, at regular intervals, fish were killed, by a blow on the head, before testes were dissected.

5. Laparotomy procedure for sex determination of fish

The fish were anaesthetized in an aerated solution of 62 mg/l tricaine methane sulfonate (MS 222) for several minutes and then placed in a nylon suspension rack. To maintain anaesthesia and oxygen supply, a tube was placed in the mouth and a solution of MS 222 (50 mg/l) was continually flushed over the gills. A ventral mid-line incision was then made, starting 0.5 cm posterior to the pectoral fins and running caudally for 5 cm. The flaps of tissue on either side of the incision were extended using retractors and a blunt probe used to examine the gonads. The males were identified by their translucent threadlike testes, each

about 8 cm long and 1 mm thick.   The ovaries in the female

trout were yellowish and were characterized by a marked

tapering from the anterior to the posterior end.   The

wound was closed with 5-0 silk sutures (Ethicon) and the

fish returned to the aquaria.   The whole procedure took

no more than 5 minutes and routinely there was 90-100%

survival.

6. Pituitary extract preparation

     Pituitaries were collected from freshly-killed, spawn-

ing chinook salmon, Oncorhynchus tschawytscha, in late Sept-

ember, at the Green River Hatchery, Auburn, Washington.

A core, containing the brain and the underlying pituitary

gland, was removed from the salmon head.   The pituitary

gland, a small spherical gland, average weight 85 mg, was

scooped out of the core, frozen immediately with solid CO_2,

and stored at -80°.   Three volumes of saline (1.25%) were

added to thawed pituitary glands and the mixture homogenized

in a Waring blender, at high speed, for 2 min.   The solution

was then centrifuged at 11,250 rpm for 15 min in a Sorvall

SS 34 rotor.   The supernatant was stored at -20° in 5 ml

aliquots.   Before injection, the thawed extract was clari-

fied by centrifugation at maximum speed on an International

bench top centrifuge for 1-2 min.

7. Cyclic nucleotide extraction

     Testes were dissected from hormonally-induced trout

at weekly intervals, over a 10-12 week testis maturation time, and immediately placed in liquid nitrogen. Samples were stored frozen at -80°. Two different procedures were used for extraction of cyclic nucleotides. For the cyclic GMP determinations, each sample was quickly weighed and placed in 1% perchloric acid (1 ml; about 5 volumes) which contained 0.5 pmol [^{3}H]cyclic GMP (10 Ci per mmol). The tissue was homogenized in a Potter-Elvjhem homogenizer, for 30 sec, and the homogenate centrifuged at 10,000xg for 10 min. The supernatant solution was adjusted to pH 7.0 with 6 N KOH and 1 M Tris.HCl, pH 7.5 added to a final buffer concentration of 0.1 M and recentrifuged at 10,000xg for 20 min. The supernatant solution was then applied to an AG 1-X8 column (0.7 x 3.5 cm), prewashed as described earlier in Methods. After sample adsorption, the column was washed with 0.1 N formic acid (10 ml) followed by 2 N formic acid (12 ml) and then 5 N formic acid (14 ml). The 5 N formic acid eluate, containing cyclic GMP (Appendix ; Figure 19A) was rotary evaporated at 36°, redissolved in distilled H_2O and re-rotary evaporated at 36°. The cyclic GMP fraction was finally redissolved in 50 mM sodium acetate, pH 6.2 (1 ml). Total recovery of [^{3}H]cyclic GMP through the extraction and purification procedure was 60-70%. Under the rotary evaporation conditions described above, cyclic GMP was not degraded, as shown by 100% recovery of [^{3}H]cyclic GMP cochromatographing with cyclic GMP on paper.

For the cyclic AMP determinations, the cyclic nucleotides were extracted by homogenization in 5% TCA (1 ml; 5 volumes) containing 0.25 pmol [^{3}H]cyclic AMP (39.8 Ci per mmol) and centrifuged at 10,000xg for 10 min.   The supernatant was extracted 4 times with diethyl ether (about 6 ml; 4 volumes). (This was a more rapid method of acid removal than the KClO$_4$ precipitation method.)   The resulting aqueous layer was applied to AG 1-X8 columns.   The resin had been prewashed with 5 N formic acid by a batch method, as described earlier in Methods.   The column was then washed with 0.1 N formic acid (10 ml) followed by 2 N formic acid (12 ml).   The 2 N formic acid eluate, containing cyclic AMP (Appendix ; Figure 19A), was rotary evaporated as described above. Total recovery of [^{3}H]cyclic AMP through the extraction and purification procedure was 60-70%.

8. Determination of cyclic nucleotide concentrations by
   radioimmunoassay

   Cyclic nucleotide concentrations were determined by the radioimmunoassay method developed by Steiner (112) using radioimmunoassay kits purchased from Collaborative Research for cyclic GMP assays and by New England Nuclear for cyclic AMP assays.   Cyclic GMP was routinely determined in the range of 0.5-10 pmol.   Cyclic AMP was determined in the range of 1-20 pmol.   All kits (except 1 from Collaborative Research) gave 40-50% maximum binding of ^{125}I-cyclic GMP or ^{125}I-cyclic AMP to their respective antibodies and

linear standard curves over the ranges detailed above.   All samples were assayed in duplicate, at 2-3 dilutions and a portion of each sample was subjected to phosphodiesterase treatment.   The conditions for the phosphodiesterase hydrolysis were 0.025 unit of beef heart cyclic nucleotide phosphodiesterase, 30 mM Tris.HCl, 6 mM $MgCl_2$ and sample incubated in a final volume of 50 μl for 20 min at 37°.   The reaction was terminated by heating in a temperature heat block at 100° for 1 min.   Samples with cyclic nucleotide "blank" values, after phosphodiesterase treatment, above 10% of the untreated sample, were not used.   About 5% of samples had unacceptable blanks.

9. Tissue preparation for cyclic nucleotide phosphodiesterase assay

Testes were excised from hormonally-induced trout and were placed on ice, weighed and minced with a scalpel.   They were then homogenized in 4 volumes of buffer, by 10 passes of a motordriven pestle in a loose-fitting glass-Teflon homogenizer.   Homogenizing buffer was 10 mM Tris.HCl, pH 7.5, 0.25 M sucrose, 4 mM 2-mercaptoethanol, 2 mM $MgCl_2$ (buffer A) for all total phosphodiesterase activities and DEAE-cellulose separations.   The homogenate was filtered through 4 layers of cheesecloth to remove connective tissue.

Sperm were collected from freshly-killed fish by applying manual pressure from the anterior abdomen toward the posteriorly located genital-anal opening.   Sperm were

homogenized in 9 volumes of buffer A and filtered through

4 layers of cheesecloth.   Total sperm activities were

expressed as pmol cyclic nucleotide hydrolyzed / min / mg

protein.   Protein in sperm suspensions was 1.3% of the

wet weight, compared with 8-12% of the wet weight

in immature testis samples, and 5-8% of the wet

weight in maturing testis.   Since it was necessary to

assay a large volume of sperm suspension, in order to make

a protein determination, the turbidity in such assays may

have increased the apparent protein concentration.

10. <u>DEAE-cellulose column chromatography of cyclic nucleotide</u>

    <u>phosphodiesterase activities</u>

     Cheesecloth-filtered homogenates of trout testes in

buffer A were directly applied to DEAE-cellulose (DE 32)

columns (0.5 x 10.5 cm or 0.8 x 22 cm) pre-equilibrated in

20 mM sodium acetate, pH 6.5, buffer containing 4 mM

2-mercaptoethanol.   After sample adsorption, columns were

washed with several column volumes of this buffer.   The

initial wash contained no phosphodiesterase activity.   A

linear gradient from 20 mM to 1 M sodium acetate, pH 6.5,

was then applied, with a flow rate of 26 ml per hour and

a total gradient volume of 200 ml.   Column fraction volume

was 4.5 ml.   (The exception to this was in Figure 11A, in

which case there was a 100 ml total gradient volume and a

2.2 ml column fraction volume.)   Each collection tube

contained 50 µl, (or 25 µl in the case of the 2.2 ml column

fraction volume) of a 0.2 M $MgCl_2$ and 100 mg/ml bovine serum albumin solution to give a final concentration in the 4.5 ml of 1.8 mM $MgCl_2$ and 0.9 mg bovine serum albumin/ml. (In the absence of this stabilizing solution, phosphodiesterase activities in DEAE-cellulose column fractions were very low.) Fractions containing phosphodiesterase activity were pooled and dialyzed against 10 mM Tris.HCl, pH 7.5, 1 mM $MgCl_2$, 0.4 mM dithiothreitol in 50% glycerol (v/v). The dialysates were aliquoted into small volumes and stored at -20° for future kinetic analyses. Fractions were stable with respect to cyclic AMP hydrolysis, but not for cyclic GMP hydrolysis, for at least 6 months.

For DEAE-cellulose chromatography of 100,000xg super-natant and sonicated 100,000xg pellet trout testis fractions (Figure 13A and B), the testes from 2 fish (630 mg/fish) were excised and homogenized in 7 ml of buffer A and then passed through 4 layers of cheesecloth. From this homo-genate, 3 ml was directly applied to a DEAE-cellulose column and the phosphodiesterase activities fractionated, 1 ml was put aside for total activity assays and the remaining 3 ml was centrifuged at 100,000xg for 1 hr. The supernatant was pipetted off and stored at -20° for future DEAE-cellulose chromatography. The 100,000xg pellet was washed with buffer A, rehomogenized and recentrifuged at 100,000xg for 1 hr. The resulting supernatant was discarded and the pellet resuspended in the original volume of buffer A. After sonication at 30 sec/ml by a Branson sonifier fitted

with a microtip (Model W 185), Plainview, N.Y., at a setting
of 5, the solubilized pellet was recentrifuged at 30,000xg
for 20 min and the supernatant pipetted off and stored at
-20° for future DEAE-cellulose chromatography.   Preparation
of DEAE-cellulose columns was as described for total testis
homogenates, except that column size was 0.8 x 16 cm, and
the total gradient volume was 100 ml.   Column fractions of
2 ml were collected.   Fraction tubes contained 25 μl of the
stabilizing solution described above.

All DEAE-cellulose columns and related operations were
carried out at 4°.

11. Cyclic nucleotide phosphodiesterase assay

The two-step assay for cyclic nucleotide phosphodiest-
erase activity was similar to that described previously (60,
103, 105) with the modifications of the use of E. coli
alkaline phosphatase, instead of snake venom nucleotidase,
for the second incubation, and a smaller assay volume of
100 μl.

An appropriate aliquot of enzyme, to obtain 10-40%
conversion of substrate to product, was incubated for 5
to 30 min at 30° in 10 mM Tris.HCl, pH 7.5 or 8.0, 2 mM
$MgCl_2$, 4 mM 2-mercaptoethanol buffer containing about
5×10^{-8} M [^{3}H]cyclic AMP or [^{3}H]cyclic GMP, in a total
volume of 100 μl.   Assays were in siliconized tubes
(10 x 1.2 cm).   When indicated, EGTA (250 μM) or Ca^{2+}
(100 μM) was included in this volume.   For higher substrate

concentrations, the indicated amount of unlabeled cyclic nucleotide was included. Chromatographic profiles were assayed at pH 8.0 for maximum assay activity. All other assays were at pH 7.5 to approximate physiological conditions. The reaction was terminated by heating in a temperature heat block at 100° for 90 sec. (For the final Table V. cyclic GMP phosphodiesterase assays, termination was for 60 sec at 100° since a lower blank was thus obtained.) Tubes were cooled on ice, re-equilibrated to 30° and 10 μl of E. coli alkaline phosphatase (0.37 mg/ml) added for a further 20 min incubation. This reaction was terminated by the addition of 1 ml of AG 1-X8 resin slurry at the appropriate pH i.e. pH 2.5 for cyclic GMP assays and pH 3.7 for cyclic AMP assays. These resin slurries were stirred with a magnetic stirrer while additions were being made. Assay tubes were left to stand for at least 10 min after resin slurry additions and then centrifuged at 3,000xg for 5 min. A 0.4 ml aliquot of the resulting supernatant solution was added to 5 ml of TTP or a 0.6 ml aliquot to 5 ml of SCINTREX scintillation fluids and the radioactivity determined.

Recoveries of nucleosides in the supernatant fraction were determined by [^{3}H]guanosine or [^{14}C]adenosine recovery and found to be 80% and 90% respectively. Resin slurries at the above pHs gave blanks of the respective unbound [^{3}H]cyclic nucleotide of 2-14% for cyclic GMP and 2-8% for cyclic AMP. Blanks were consistent within assays to $\pm$ 0.5%,

but varied between assays with increasing blanks correlating
to increasing length of time after purification of the [^{3}H]
cyclic nucleotides on AG 1-X8 columns.   Due to the low
cyclic GMP phosphodiesterase activities in column fractions
after DEAE-cellulose separations, a combination of 0.2 ml
of 95% ethanol plus 0.8 ml of AG 1-X8 resin slurry was used
to terminate cyclic GMP assays, since the recovery of guano-
sine under these conditions was 90-95%, while the cyclic GMP
blank was unchanged.   Duplicates were less consistent with
the addition of alcohol, so total cyclic GMP activities were
measured using the 1 ml of AG 1-X8 resin slurry at pH 2.5,
to terminate the second reaction, and activities were corr-
ected for the 80% guanosine recovery.   Cyclic AMP activities
were also corrected for the 90% adenosine recovery.

12. Tissue preparation for guanylate cyclase assay

     Trout testes were homogenized in 10 volumes (2-3 ml)
of 10 mM Tris.HCl, pH 7.5, 0.25 M sucrose, 4 mM 2-mercapto-
ethanol and 1 mg/ml bovine serum albumin (buffer B).
Homogenization conditions were the same as those described
for cyclic nucleotide phosphodiesterase activities.   The
homogenate was centrifuged at 100,000xg for 1 hr at 4°, and
the supernatant pipetted off and stored frozen at -20°.
The pellet was resuspended in 1 ml of buffer B and this was
then centrifuged at 100,000xg for 1 hr at 4°.   The second
supernatant was discarded and the final pellet resuspended
in 1 ml of buffer B and stored frozen at -20°. Before

guanylate cyclase assay, the thawed pellet suspension was homogenized as described above.

13. Guanylate cyclase assay

The incubation mixture for the assay of guanylate cyclase activities contained 50 mM Tris.HCl buffer, pH 7.5, 2 mM $MnCl_2$, 1.6 mM MIX, 1 mM cyclic GMP, 0.1-0.25 mM $[\alpha^{32}P]$ GTP (specific activity 10-30 cpm per pmol; 300,000-400,000 cpm per assay tube), 15 mM phosphocreatine, 12.25 units of creatine phosphokinase, $[^{3}H]$cyclic GMP (25 nCi; 13,000 cpm per assay tube) and 40-250 µg of testis protein, in a final volume of 100 µl.   When indicated, 1 µl of Triton X-100 was added to make a final concentration of 1% Triton X-100, or, 15 µl of salmon pituitary extract was added.   The assay blank value was obtained using incubation mixtures without testis protein.   Incubations were carried out at 37° for various lengths of time (10-30 min).   The reaction was initiated by the addition of testis protein and was terminated by the addition of 20 µl of a solution of EDTA (30 mM) to attain a final concentration of 5 mM.   The reaction mixture was diluted to 0.5 ml with distilled H_2O, vortex mixed and transferred to a BioRad AG 50W-X2, 200-400 mesh, hydrogen form, resin column (0.7 x 4 cm) prepared by pipetting 2 ml of a 50% v/v suspension of the resin in H_2O into the column.   After adsorption of the 0.5 ml, the column was eluted with 0.4 ml of H_2O and the 0.9 ml total initial eluate added to 5 ml of SCINTREX for radioactivity

determination. About 40-60% of the $[\alpha^{32}P]$GTP was recovered in this fraction. The column was then eluted with an additional 1 ml of H_2O and this eluate chromatographed directly onto a neutral alumina (Woelm, activity grade I) column (0.7 x 2.5 cm) made up by pipetting 2 ml of a 50% v/v suspension of neutral alumina in 0.1 M Tris.HCl, pH 7.4, into the column. The alumina column was then eluted with 4 ml of 0.1 M Tris.HCl, pH 7.4. Eluates were added to 10 ml of SCINTREX and radioactivity (both $[^{32}P]$ and $[^3H]$) was determined. All results were corrected for the recovery of $[^3H]$ cyclic GMP and were calculated as pmol cyclic GMP formed from the specific activity of the GTP used in each experiment. After dilution of $[\alpha^{32}P]$GTP with unlabeled GTP, the final GTP concentration was determined by absorbance at 252 nm, using an extinction coefficient of 13,800 M^{-1}. The overall recovery of cyclic GMP, after losses during incubation and the two stage column procedure, was 45-65%. The blank of the assay, under these conditions, ranged from 0.006-0.05% of the added $[\alpha^{32}P]$GTP.

The above assay is a slight modification of a previously reported assay (106). In the latter case, the first column eluate was 1 ml (compared with 0.9 ml above) and the final 0.1 M Tris.HCl eluate was 2 ml (compared with 4 ml above). The slight modifications were made in the present research because the overlap of GTP and cyclic GMP was greater on the AG 50W-X2 columns prepared by the present author, and also the majority of $[^{32}P]$cyclic GMP was found in the 3rd and 4th

milliliter off the neutral alumina column (this includes
the first milliliter adsorbed from the AG 50W-X2 column
onto the neutral alumina column).

14. <u>Protein assay</u>

Protein concentrations were assayed by the method of
Lowry et al (107) with bovine serum albumin as the standard.

RESULTS

A. Testes growth rate during hormonally-induced spermatogenesis in trout

   A testes weight doubling time of about 1 week is observed in rainbow trout, Salmo gairdnerii, injected twice weekly with a salmon pituitary extract (10). This results in a logarithmic testes weight increase during the first 8 to 10 weeks of testicular maturation. However, the growth rate of hormonally-induced testes is not completely synchronous from individual to individual during development (Table I). Also, there are large variations in initial testes weights between different batches of trout. Thus, zero time testes weights (sum of the weight of both testis) varied between about 30 and 130 mg. This appeared to be related to the age of the fish, or the season in which they were obtained, or to the length of time they were kept in the laboratory aquaria before the start of experiments. Batches of trout with large zero time testes produced proportionally large testes throughout development.

   Figure 3 shows the response to hormone injection of a batch of trout whose initial testes weights were in the range of 30 to 60 mg. The plateau and loss of weight at the end of the growth period is a consequence of germ cell cytoplasmic loss and testicular tissue degeneration in the final stages of spermatogenesis. The relationship between duration of hormone treatment and testes weight permits the reporting of

FIGURE 3

Testes growth rate during hormonally-induced

spermatogenesis in trout

Weights of testes (i.e. weight of a pair of testes) from

rainbow trout, Salmo gairdnerii, maintained under the

conditions described in Materials and Methods, are plotted

as a function of days following the initiation of twice

weekly injections of salmon pituitary extract.

An average fish weighed about 250 gm and was about 26 cm

long.

Each point represents an average weight of the pair of

testes from a sampling of 2 or 3 fish.
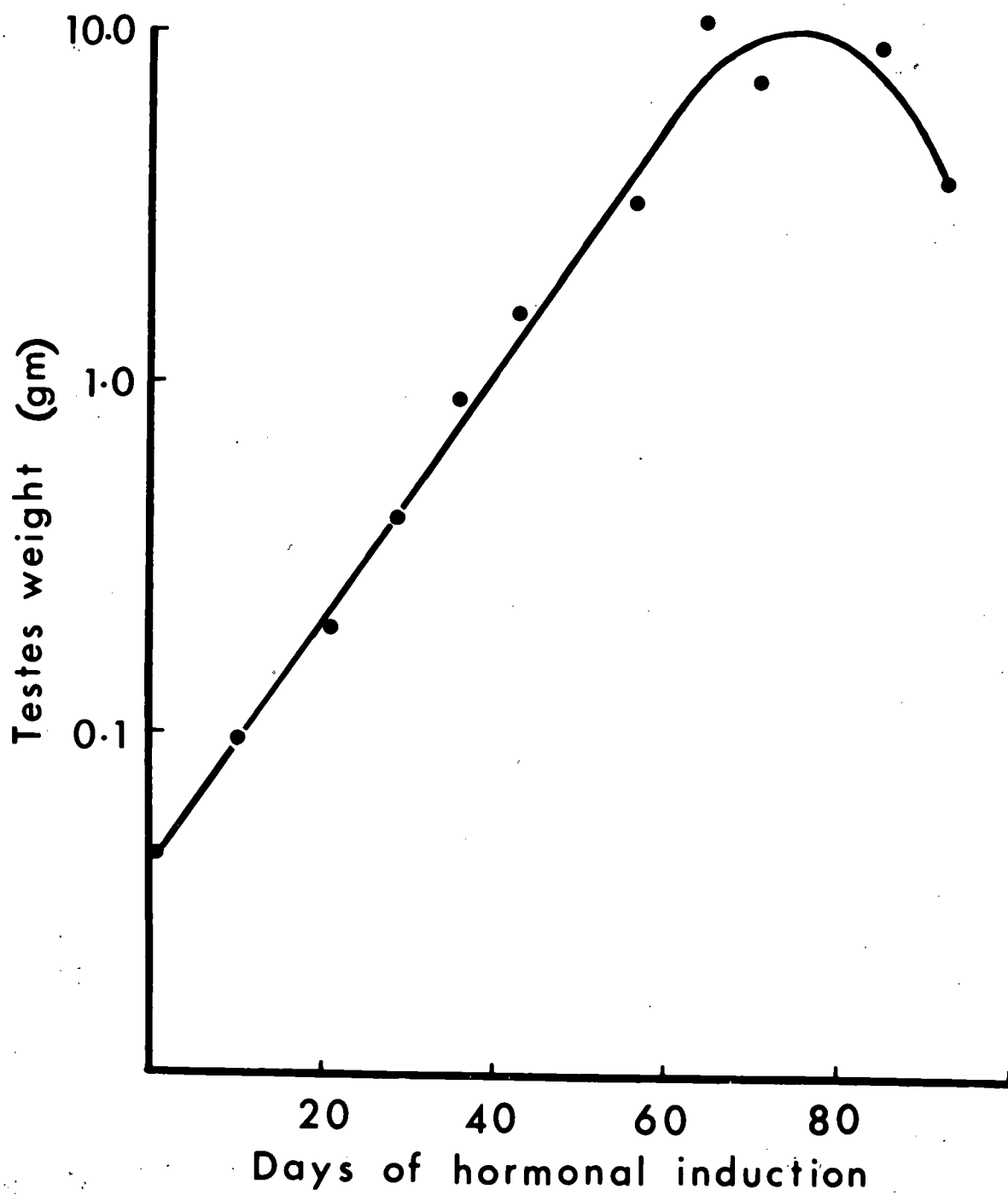
changes in cyclic nucleotide biochemistry during development with reference to either time or testes weight.

B. Cyclic GMP and cyclic AMP concentrations in trout testis during spermatogenesis

Cyclic nucleotides have been determined by receptor protein binding displacement (108), enzymatic cycling (109), protein kinase activation (110), high pressure ion exchange chromatography (111), and radioimmunoassay (112). In initial experiments (Appendix) , high pressure ion exchange chromatography was investigated, but was rejected due to its inability to detect the amount of cyclic GMP available from trout testis. Subsequently, radioimmunoassay was found to be a particularly sensitive and convenient technique, and was used in all measurements of cyclic GMP and cyclic AMP reported in this thesis.

The cyclic GMP in trout testis was determined in 3 experimental batches of hormonally-induced rainbow trout. Testes were rapidly excised and frozen in liquid nitrogen to minimize post-mortem alteration of the cyclic nucleotide concentration. In the first experiment, nucleotide extracts were not purified before cyclic GMP was measured. However, after treatment with beef heart cyclic nucleotide phosphodiesterase, some cyclic GMP samples had high radioimmunoassay blank values (greater than 10% of the untreated sample). Therefore, in subsequent experiments, cyclic GMP extracted from testis was purified before radioimmunoassay.

From the available purification techniques for cyclic nucleotides from biological sources,i.e. adsorption chromatography on neutral alumina (113), thin-layer chromatography on PEI-cellulose (114), inorganic salt coprecipitation using $ZnCO_3$ and $BaSO_4$ (115), or ion exchange chromatography on cation exchange resins, such as DOWEX 50 (116), and anion exchange resins, such as DOWEX 1 and 2 (117), anion exchange was used in this research because of its relative ease and speed. In all experiments subsequent to the first, trout testis nucleotide samples were purified by anion exchange chromatography on AG 1-X8 columns, as described in Materials and Methods.

Only a limited number of samples were assayed in the first experiment (data not shown), but since high cyclic GMP concentrations (1 μmol per kg testis) were observed early in development and not in mature testis, a more thorough sampling of testes at early stages of development was made in the second experiment (Table I and Figure 4). In experiment 2, cyclic GMP was found to be elevated in zero time testis and in testis during premeiotic proliferation of spermatogonia (Figure 4). Prior to the time of the meiotic reduction division in trout testis, an abrupt 10 fold decrease in testis cyclic GMP was observed (Figure 4). The onset of the meiotic reduction division in hormonally-induced trout testis, at between 5 to 6 weeks after the initiation of hormone injection, was determined in a study of predominant cell types during development, their DNA content and their [3H]thymidine

TABLE I

CYCLIC GMP IN TROUT TESTIS DURING HORMONALLY-INDUCED

SPERMATOGENESIS (EXPERIMENT 2)

A. Samples from testes with an average weight for the

time of hormonal induction

Day of Hormonal Induction	Total Testes Wet Weight (mg)	Cyclic GMP[a] (μmol/kg wet weight)	Cyclic GMP[a] (pmol/mg protein)
0	37	1.93	19
	56	1.45	13
10	92	1.00	10
	93	1.93	20
15	100	1.51	19
24	300	1.12	13
30	500	0.39	4.0
39	880	0.12	1.3
45	1400[b]	0.08	0.8
	1400[c]	0.08	0.9
70	7000[b]	0.06	1.1
	7000[c]	0.04	0.7
87	9200[b]	0.04	0.8
	9200[c]	0.03	0.6
98[d]		0.15	12[e]

B. Samples from testes with an abnormal weight for the

time of hormonal induction

15	400	0.30	3.1
24	780	0.14	1.4
30	150	0.81	12
39	160	0.85	10

a
   Each sample assayed for cyclic GMP in duplicate, at
   2-3 dilutions, in 2 radioimmunoassays and the mean
   value of these results recorded.   Duplicate range ±10%.
   Overall range for the same sample at different dilutions
   was ±30%
b,c
   Two samples from the same testes, assayed as above
d
   Sperm
e
   Assuming a 1.3% protein content, as in other sperm samples

FIGURE 4

Comparison of cyclic GMP concentration and testes weight
during hormonally-induced spermatogenesis in trout

Cyclic GMP extracted and assayed as described in Materials
and Methods, was measured in testis from rainbow trout,
Salmo gairdnerii, grown as described in the legend for
Figure 3.   The average wet weight growth curve is as in
Figure 3.

Values for cyclic GMP were obtained from testes with an
average wet weight for the time of hormonal induction.
Each biological sample was assayed in duplicate, at 2-3
dilutions, in 2 radioimmunoassays and the mean value of
the sum of these results recorded.   The range between
duplicates in the same radioimmunoassay was $\pm$ 10%.
The overall range between values obtained for the same
biological sample at different dilutions was $\pm$ 30%.
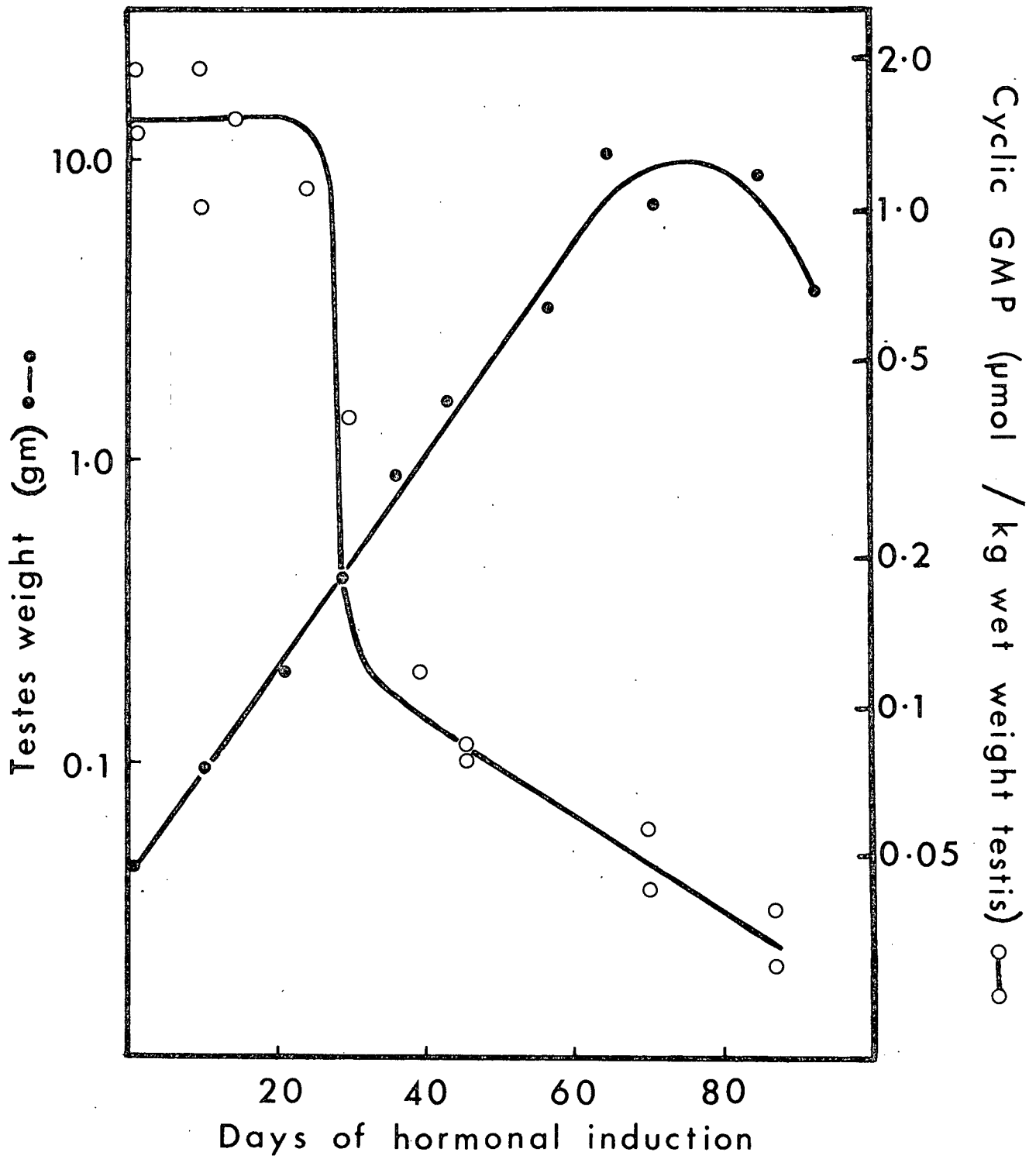
incorporation (10). Although the exact timing of meiosis
was not determined in the present studies, it is obvious that
the abrupt decrease in cyclic GMP occurred before the prolif-
eration of germ cells had ceased (Figure 4). During the stages
following the large decrease in cyclic GMP, an inverse corr-
elation between testes weight and cyclic GMP concentration was
observed (Table IA and Figure 4). Only those values for
cyclic GMP obtained from testes with the average weight for
the time of hormonal induction were used in Figure 4 . In
testes which were not developing at the average growth rate, as
shown in Figure 3, cyclic GMP concentrations again showed an
inverse relationship to total testes wet weight (Table IB).

The cyclic GMP concentration observed in sperm of
0.15 μmol/kg wet weight is similar to a previous report of
cyclic GMP in trout sperm of 0.2-0.3 μmol/kg wet weight (129).
The difficulties in obtaining an accurate protein concentrat-
ion in sperm suspensions (Materials and Methods) puts doubt
on the expression of cyclic GMP in pmol/mg protein.

In experiment 2, rather large variations were observed
in cyclic GMP in early developmental samples from testes of
approximately the same total wet weight (Table I). Such
variations were also observed in early developmental samples
in the third experiment (Table II). In experiment 3, cyclic
GMP was found to be elevated in all samples from zero time
to 4 weeks of hormonal induction (Table II), and slightly
higher than cyclic GMP in early samples in experiment 2.
(Table I). In experiment 3, cyclic GMP again decreased

TABLE II

CYCLIC GMP IN TROUT TESTIS DURING HORMONALLY-INDUCED

SPERMATOGENESIS (EXPERIMENT 3)

Day of Hormonal Induction	Total Testes Wet Weight (mg)	Cyclic GMP[a] (μmol/kg wet weight)
0	30	2.36
21	130	3.20
28	110	1.80
28	340	2.20
35	910	0.26
49	630	0.38
63	16000	0.03
66	10000	0.08

[a] Each sample assayed in duplicate, at 2 dilutions, in 2 radioimmunoassays and the mean value of these results recorded. Duplicate range ± 10%. Overall range for the same sample at different dilutions was ± 30%.

dramatically after about 4 to 5 weeks of hormone injections
(Table II). The concentration of cyclic GMP again decreased
to about one-tenth of its original concentration. During
the following stages of testis development, there was an
inverse correlation between testes weight and cyclic GMP
concentration (Table II).

In experiment 3, testis cyclic GMP was compared with cyclic
GMP in another trout tissue, namely the liver. Liver cyclic
GMP was measured in both immature (3 weeks of hormonal induction)
and mature (12 weeks of hormonal induction) fish, and found to
be 0.22 $\pm$ 0.07 and 0.11 $\pm$ 0.01 μmol/kg wet weight liver,
respectively. It is not clear if this 100% reduction in trout
liver cyclic GMP is significant. The trout liver cyclic GMP
concentrations are somewhat higher than those observed in rat
liver of 0.04-0.07 μmol/kg wet weight liver (128).

In experiment 4, cyclic AMP was determined in trout testis
during hormonally-induced spermatogenesis (Figure 5). Cyclic
AMP decreased 2 fold at a premeiotic stage of testis develop-
ment, at about, or prior to the time of the 10 fold decrease
in cyclic GMP (Figure 4). Cyclic AMP and cyclic GMP conc-
entrations in zero time testis were approximately equal at
about 2 μmol/kg wet weight testis (Figures 4 and 5). After
the 2 fold premeiotic decrease in trout testis cyclic AMP, no
significant changes in cyclic AMP concentrations in developing
testis occurred during the remainder of spermatogenesis (Figure 5).

FIGURE 5

Cyclic AMP concentrations in testis during hormonally-
induced spermatogenesis in trout

Cyclic AMP, extracted and assayed as described in Materials

and Methods, was measured in testis from rainbow trout,

Salmo gairdnerii, grown as described in the legend for

Figure 3.

Values for cyclic AMP were obtained from testes with an

average wet weight for the time of hormonal induction.

Each point represents a biological sample assayed in

duplicate, at 2-3 dilutions, in 2 radioimmunoassays and

the mean value of the sum of these results recorded.

The range between duplicates in the same radioimmunoassay

was $\pm$ 10%.   The overall range between values obtained for

the same biological sample at different dilutions was $\pm$ 30%.
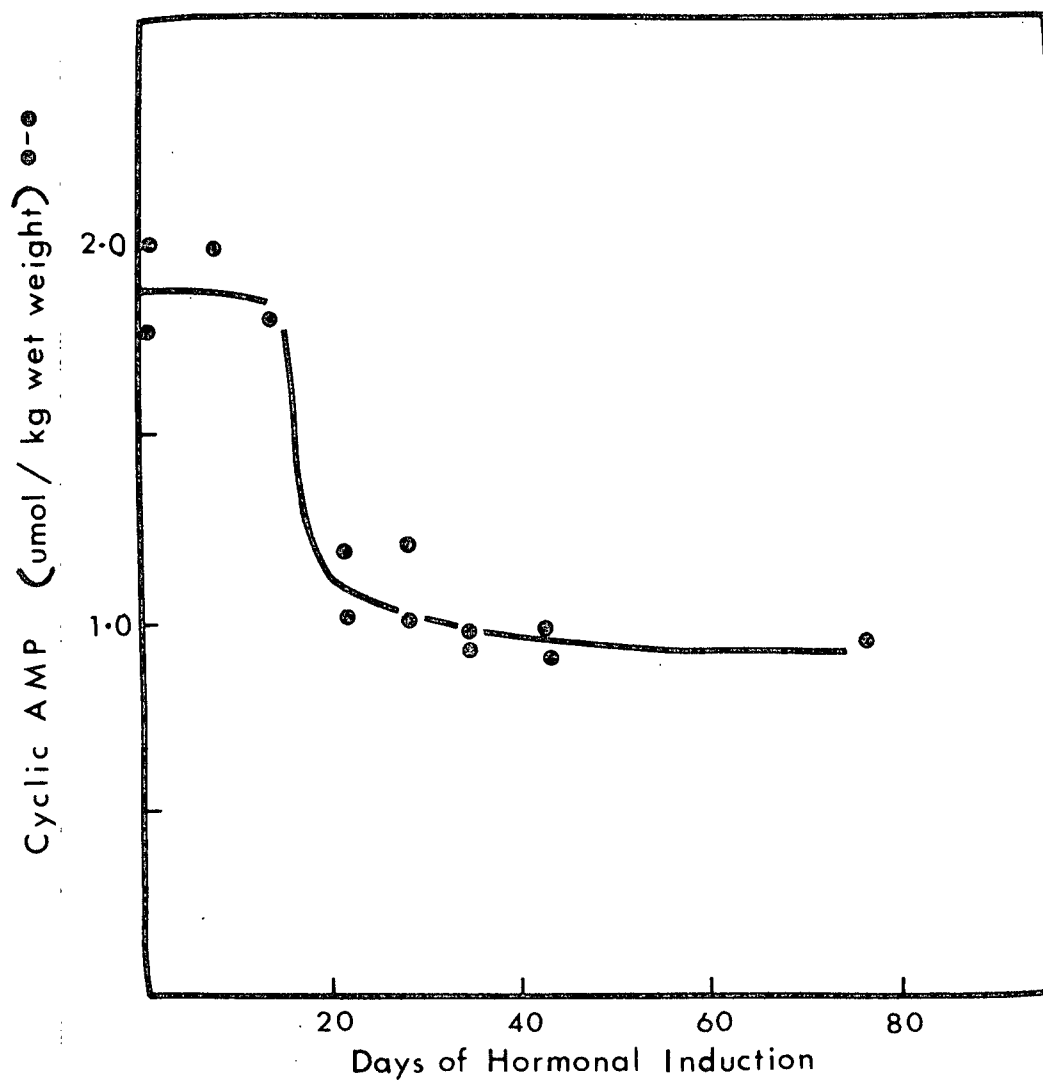
C. Cyclic AMP and cyclic GMP phosphodiesterase activities
   in trout testis during spermatogenesis

1. Assay system

   A radioisotope assay system provides the maximum

sensitivity for cyclic nucleotide phosphodiesterase assays

(118) and was therefore used in this research.   In this

method, [^{3}H]cyclic nucleotide is hydrolyzed to the corres-

ponding 5'-nucleotide, which is then converted to a nucleo-

side by the addition of excess phosphatase.   In many cases

(60, 103, 105) the excess 5'-nucleotidase present in snake

venom has been utilized in this second incubation.   In the

present research, E. coli alkaline phosphatase was used.

Separation of nucleoside from cyclic nucleotide can be

accomplished by passage over an alumina column (113), a

DOWEX 1 or 2 column (119, 120), by thin-layer chromato-

graphy (121), by paper chromatography (122) or by batch use

of DOWEX 1 resin (123).   The latter method, modified by

acidification of the resin prior to its use, resulted in

quantitative recoveries of adenosine, guanosine and their

major metabolites (103, 105).   This assay system provides

a simple, rapid and reliable method by which cyclic AMP or

cyclic GMP phosphodiesterases can be determined, even in

crude tissue preparations, and, was thus adopted for use.

   The temperature of 30° for activity assays was chosen

for comparison of results with previous cyclic nucleotide

phosphodiesterase studies in fish sperm (88).

DEAE-cellulose profiles were assayed at pH 8.0 for maximum assay activity. All other assays were at pH 7.5. Following assay conditions previously described (119), a Mg^{2+} concentration of 2 mM was used. When included, EGTA was present to eliminate the possible effects of varying amounts of Ca^{2+}-dependent phosphodiesterase protein activator in tissue preparations. When included, Ca^{2+} was present to investigate the effects of such an activator. The assay buffer contained 2-mercaptoethanol because it had been shown to stabilize the enzyme activity from other tissues (124).

2. Properties

Cyclic AMP and cyclic GMP phosphodiesterases in trout testis were stable, at the assay temperature of 30°, for at least 30 min, at 0.1M substrate, as shown in Figure 6A. In one testis homogenate, a study of enzyme requirement for Mg^{2+}, showed an approximate Mg^{2+} optimum of 8-10 mM and 2-10 mM for cyclic AMP and cyclic GMP phosphodiesterase activities, respectively (Figure 6B). A broad pH optimum from pH 7.5 to 8.0, for both cyclic AMP and cyclic GMP phosphodiesterase activities, was determined in one trout testis homogenate (Figure 7B). In the same testis homogenate, (Figure 7A) EGTA was shown to reduce both cyclic AMP and cyclic GMP phosphodiesterase activities, measured at micromolar subtrate, by about 20%. Due to the complexities of multiple enzyme forms, detailed developmental studies of Mg^{2+} optima and pH optima were not made.

FIGURE 6

A. Stability of phosphodiesterase activities at 30°

Phosphodiesterase activities, measured in a homogenate of

testis, from trout which had been injected with a salmon

pituitary extract for 8 weeks and maintained under conditions

described in Materials and Methods.   Total testes weight

was 4g.   Phosphodiesterase activities were assayed, at 30°,

in standard incubation mixtures at pH 7.5, as described in

Materials and Methods.   Cyclic nucleotide concentration was

0.1 mM.   EGTA was not included in assays.   ●—● cyclic AMP

  O—O cyclic GMP

B. Phosphodiesterase activities as a function of Mg^{2+}

   concentration

Phosphodiesterase activities, in the homogenate described

above, were assayed, at 30°, in standard incubation mixes

at pH 7.5, with varying concentrations of Mg^{2+}, as described

in Materials and Methods.   Cyclic nucleotide concentration

was 0.1 mM.   EGTA was not included.   Symbols as above.

57.
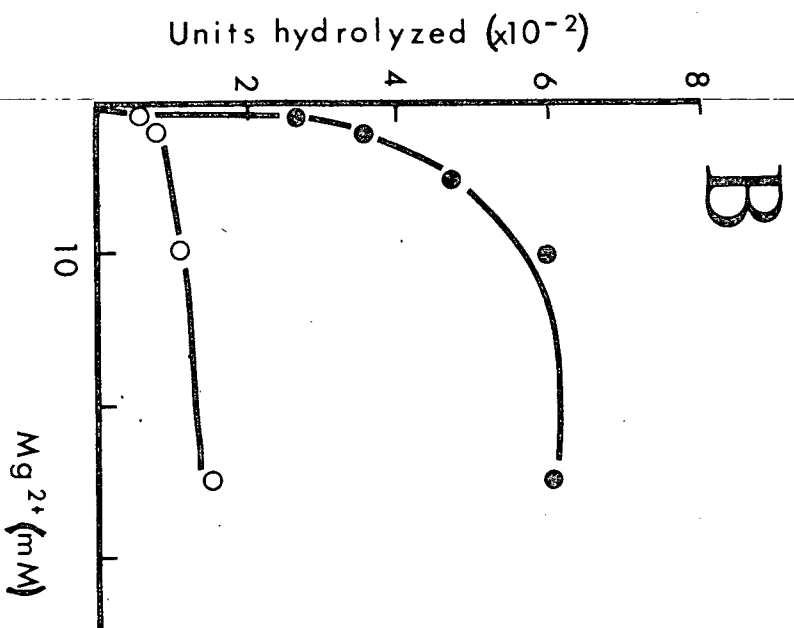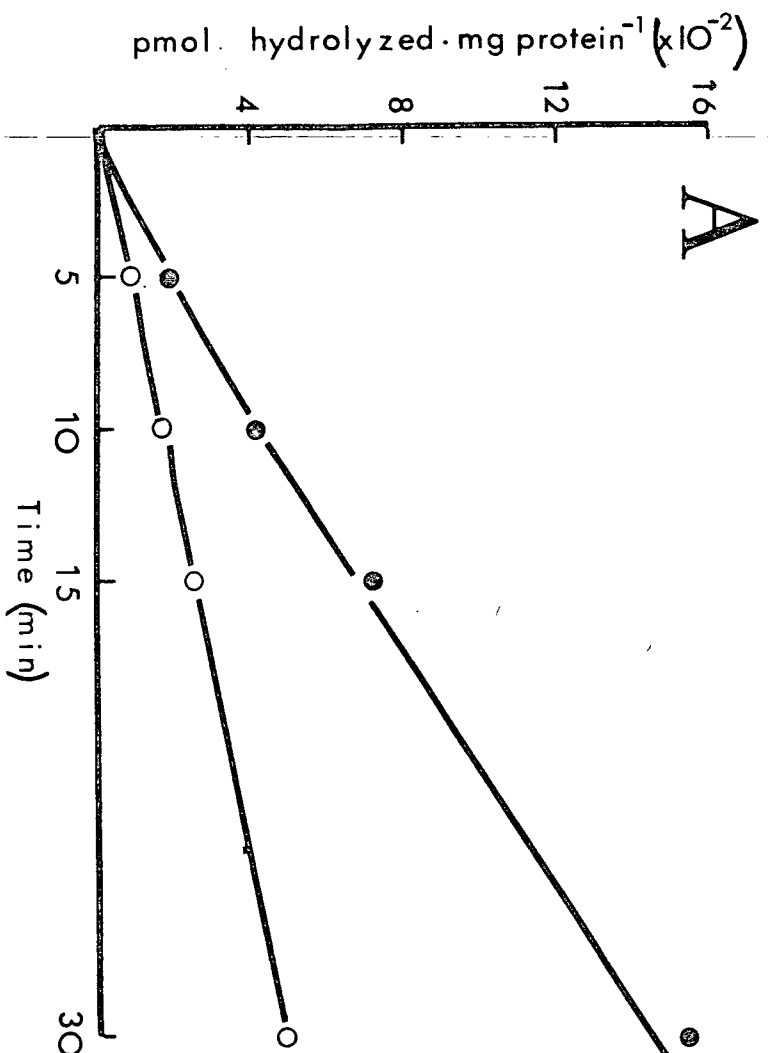
FIGURE 7

A. Phosphodiesterase activities in the presence or absence

  of EGTA

Phosphodiesterase activities, measured in a testis homogenate,

from trout which had been injected with a salmon pituitary

extract for 4 weeks and maintained under conditions described

in Materials and Methods.   Total testes weight 629 mg.

Phosphodiesterase activities were assayed, at 30°, in

standard incubation mixtures at pH 7.5, as described in

Materials and Methods.   Cyclic nucleotide concentration was

1 µM.   When present, EGTA concentration was 250 µM.

> ▲ — ▲ cyclic AMP
>
> ● — ●. cyclic AMP plus EGTA
>
> △ — △ cyclic GMP
>
> ○ — ○ cyclic GMP plus EGTA

B. Phosphodiesterase activities as a function of pH

Phosphodiesterase activities, in the homogenate described

above, were assayed, at 30°, in standard incubation mixtures,

at varying pHs, as described in Materials and Methods.

Cyclic nucleotide concentration was 1 µM.   EGTA (250 µM)

was included in all assays.   Symbols as above.
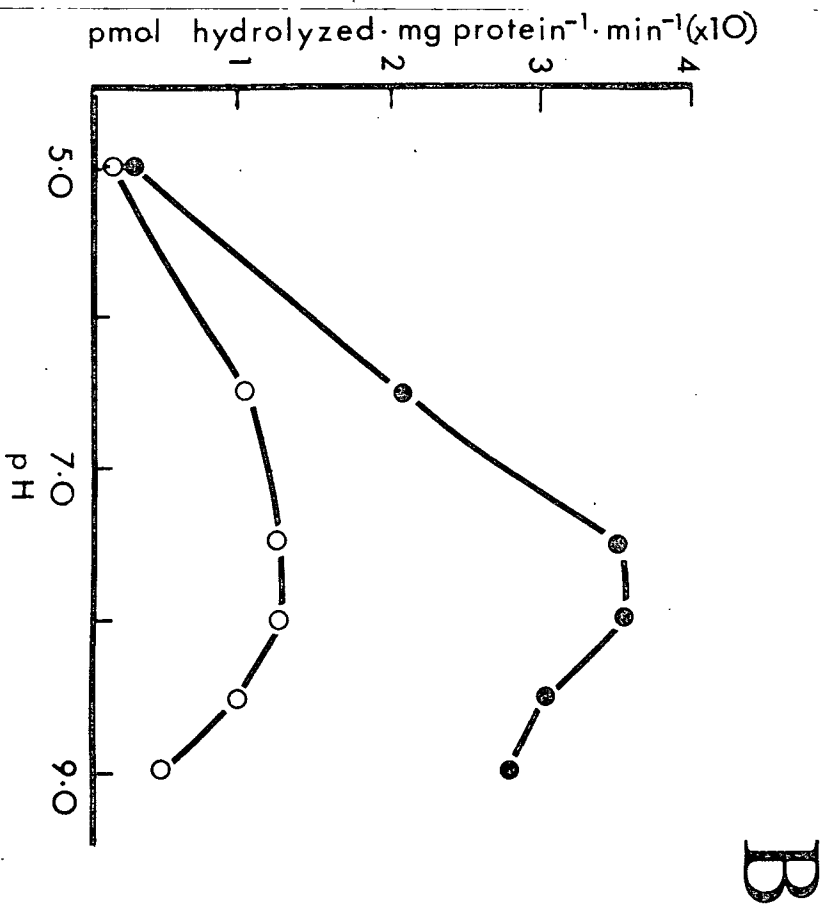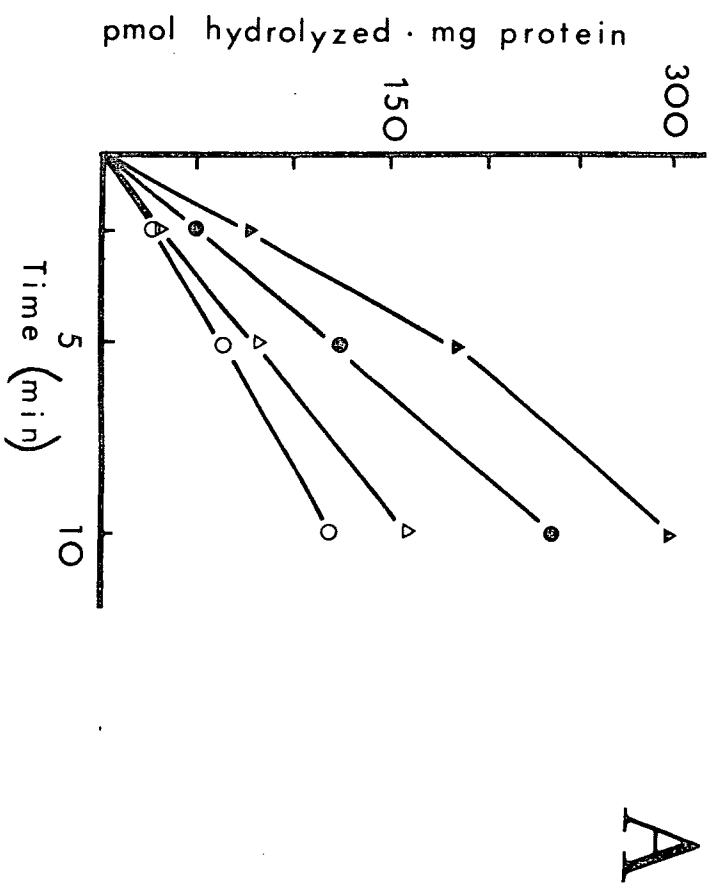
The affinities of phosphodiesterase activities for
cyclic AMP and cyclic GMP were determined in a mature trout
testis homogenate (Figures 8 and 9). Hofstee plots showed
only high affinity cyclic AMP activities (Figure 8) and both
a high and low affinity cyclic GMP activity (Figure 9).

3. Activities during testis development

Activities were measured throughout development at both
saturating (millimolar) and subsaturating (micromolar) substrate
concentrations, to detect total and high affinity components,
respectively. Total cyclic AMP phosphodiesterase activities
measured in the presence or absence of EGTA (Table III) decr-
eased 50% prior to meiosis. This was followed by an increase
during spermatid differentiation raising total cyclic AMP
phosphodiesterase activity to slightly above that observed in
immature testis. EGTA decreased total cyclic AMP activities
about 20-40% in zero time and mature testis but had an insigni-
ficant effect on activities in testis just prior to meiosis.

Total cyclic GMP phosphodiesterase activities remained
relatively constant throughout development, in the absence
of EGTA (Table III). The presence of EGTA appeared to
enhance cyclic GMP phosphodiesterase activities in immature
testis about 30%, but throughout the following stages of testis
development resulted in about a 20% decrease in cyclic GMP
activities. The rather minor effect of EGTA on both cyclic
AMP and cyclic GMP phosphodiesterase activities did not show
any important changes during development in the concentration
or the activity of a Ca^{2+}-dependent phosphodiesterase protein

FIGURE 8

Hofstee plot of the rate of cyclic AMP hydrolysis by a

testis homogenate (minus the 1,000xg pellet) from trout

hormonally-induced for 10 weeks, as described in Materials

and Methods.   Total testes weight 9.8 g.   Homogenates

were stored frozen at -20° for a week before kinetic analysis.

Homogenates were centrifuged at 1,000xg for 10 min after

thawing.   The insoluble lipid pellet was discarded and the

supernatant assayed for activity.   The unit of velocity is

nmol cyclic AMP hydrolyzed/min/mg protein.   Assays were at

30°, in standard incubation mixtures at pH 7.5, as described

in Materials and Methods.   EGTA (250 µM) was included in

all assays.   Substrate concentrations varied from 0.1-500 µM.

app km ≅ 1·1uM

app km ≅ 0·3 μM

63.
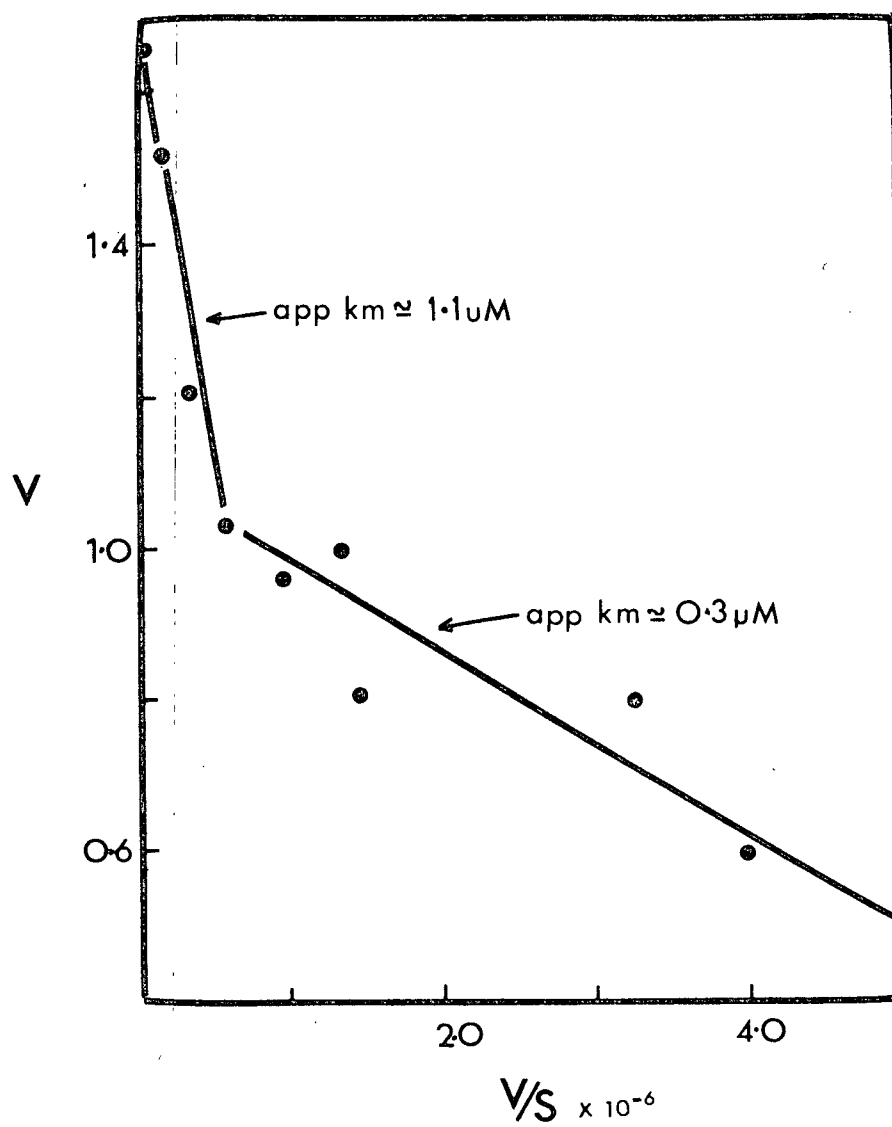
FIGURE 9

Hofstee plot of the rate of cyclic GMP hydrolysis by the

homogenate described in the legend for Figure 8.   The

unit of velocity is nmol cyclic GMP hydrolyzed/min/mg protein.

Assays were at 30°, in standard incubation mixtures at pH 7.5,

as described in Materials and Methods.   EGTA (250 μM) was

included in all assays.   Substrate concentrations varied

from 5 - 1,000 μM.
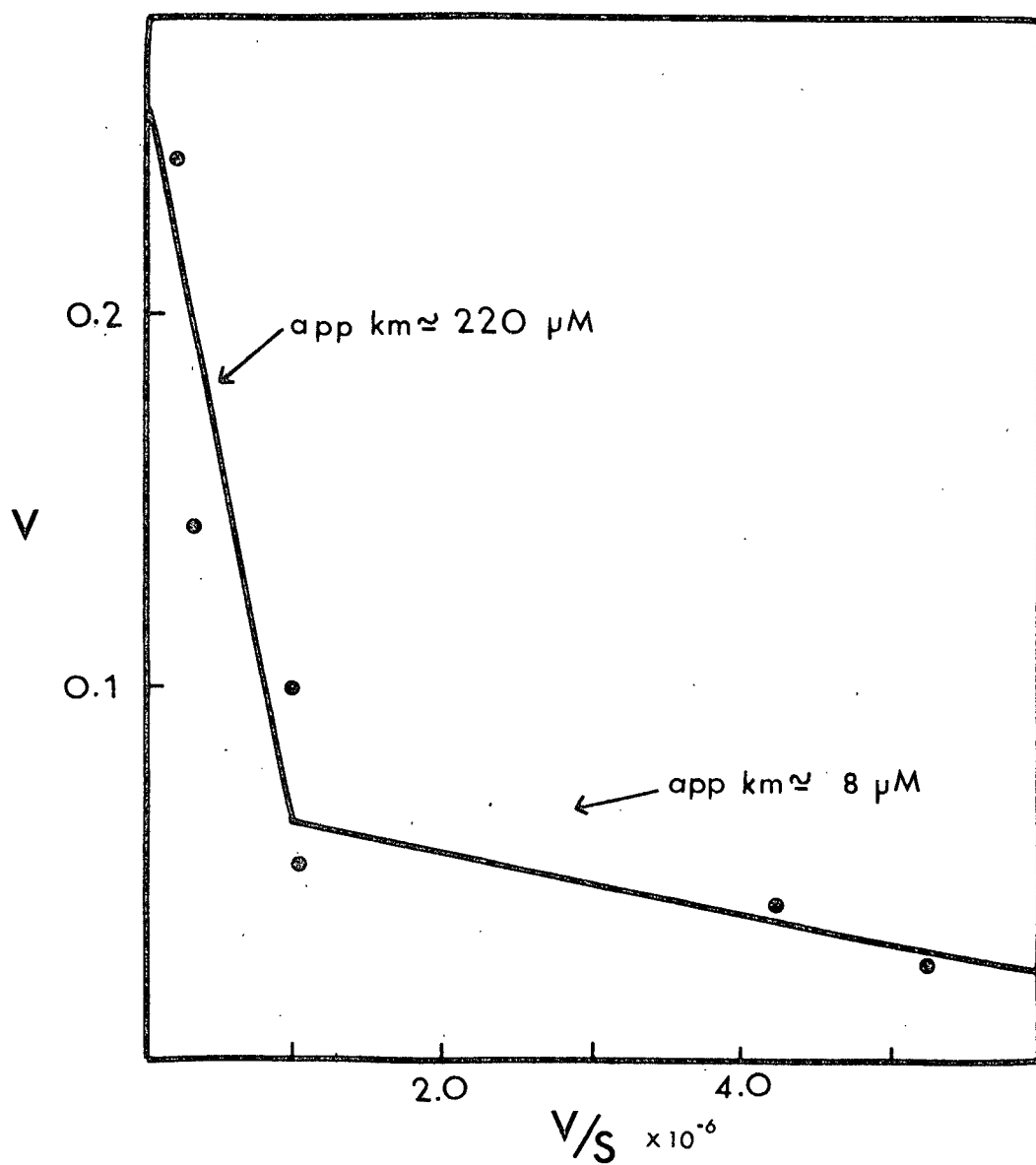
TABLE III

TOTAL CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES IN

TROUT TESTIS DURING HORMONALLY-INDUCED SPERMATOGENESIS

Week of Hormonal Induction	Total Testes Wet Weight (mg)	Cyclic AMP Activity		Cyclic GMP Activity	
		Minus EGTA [b]	Plus EGTA [c]	Minus EGTA [b]	Plus EGTA [c]
0	70	1210	1010	570	740
2	80	1390	1260	600	910
3	480	650	630	450	490
4	890	930	850	520	500
5	1500	770	560	430	350
6	3000	1000	510	510	300
8	4100	1560	1060	630	590
10	9800	1720	1210	560	480
12 [a]			450		90

Trout testes were excised, placed in liquid nitrogen and
stored at -80° until one complete developmental series of
testes had been collected. Trout were hormonally-induced
by a twice weekly injection of a salmon pituitary extract,
as described in Materials and Methods. Crude homogenates
of the thawed and weighed testes were prepared in buffer A
and assayed for cyclic AMP and cyclic GMP phosphodiesterase
activities, at 30°, in standard incubation mixtures at
pH 7.5, as described in Materials and Methods. Millimolar
substrate concentrations were used. Activity units are
pmol cyclic nucleotide hydrolyzed/min/mg protein. Values
are the means of duplicates (± 10%) from the same homogenate.

[a] This sample consisted of sperm, obtained as described in
   Materials and Methods

[b] No EGTA in assay mixture

[c] EGTA (250 μM) in assay mixture

activator in trout testis.

The specific activities of sperm cyclic AMP and cyclic GMP phosphodiesterases, measured at millimolar substrate concentrations, are respectively 3 and 6 fold lower than the specific activities in mature testis (Table III). Direct comparison between the cyclic nucleotide phosphodiesterase specific activities in sperm and in testis tissue is possibly invalid due to the difficulty in measuring protein in sperm suspensions (Materials and Methods).

Cyclic AMP and cyclic GMP phosphodiesterases, in testis homogenates from trout at different stages of spermatogenesis, were measured at micromolar substrate, in the presence of EGTA (Table IV). As seen in the total cyclic AMP activities (Table III), a 50% decrease of the small amount of initial cyclic AMP phosphodiesterase, measured at micromolar substrate, occurs in a premeiotic stage of spermatogenesis. This is followed by a 20 to 40 fold increase in cyclic AMP activity, measured at micromolar substrate, during the later stages of development (Table IV). Cyclic AMP phosphodiesterase activities were not measured at micromolar substrate throughout development in the absence of EGTA. As seen in Figure 7A, EGTA caused a 20% reduction in cyclic AMP phosphodiesterase activity, measured at micromolar substrate, in a testis homogenate from one stage of development. It is possible that, in the absence of EGTA, a slightly greater increase in high affinity cyclic AMP phosphodiesterase activity would be observed during spermatogenesis.

TABLE IV

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES MEASURED

AT MICROMOLAR SUBSTRATE IN TROUT TESTIS DURING HORMONALLY-

INDUCED SPERMATOGENESIS

Week of Hormonal Induction	Total Testes Wet Weight (mg)	Cyclic AMP Activity	Cyclic GMP Activity	A/G Ratio[a]
2	220	48	20	2.4
3	250	43	17	2.5
4	630	26	14	1.9
6	7100	430	16	27
10	9800	1000	11	91
12[b]		310	3	124

Legend as in Table III, except assays were measured at micromolar substrate concentrations. Assay mixtures all contain EGTA (250 μM). Activity units are pmol cyclic nucleotide hydrolyzed/min/mg protein.

[a]The ratio of cyclic AMP to cyclic GMP phosphodiesterase activity

[b]This sample consisted of sperm, obtained as described in Materials and Methods

Cyclic GMP phosphodiesterase activity, measured at micromolar substrate, in the presence of EGTA, decreases less than 50% progressively throughout spermatogenesis (Table IV). The ratio of cyclic AMP to cyclic GMP hydrolysis, measured at micromolar substrate, changes from about 2 to 124 during testis differentiation. This indicates the specific induction of a high affinity cyclic AMP phosphodiesterase in a late stage of spermatogenesis. In a study by Drummond and his associates, in which cyclic nucleotide phosphodiesterase activities in salmon sperm were measured, at micromolar substrate concentrations, a cyclic AMP to cyclic GMP activity ratio of 124 was also observed (88).

The sequential changes in cyclic nucleotide phosphodiesterase activities, during spermatogenesis in trout, are graphed in Figure 10. Total (Figure 10A) and high affinity (Figure 10B) phosphodiesterase activities, in the presence of EGTA, are shown both as a function of time of hormonal induction and in relation to the stage of germ cell differentiation. The induction of a high affinity cyclic AMP phosphodiesterase, at about meiosis, is evident in Figure 10B.

Since it had been shown that Ca^{2+}, in the presence of a Ca^{2+}-dependent phosphodiesterase protein activator, increases the activity of cyclic GMP hydrolysis more so than cyclic AMP hydrolysis (74), a more detailed study of the effects of Ca^{2+} and EGTA on high affinity cyclic GMP phosphodiesterase activities in developing trout testis, was undertaken. The results of this study are summarized in Table V. Only in

FIGURE 10

A. <u>Total cyclic nucleotide phosphodiesterase activities in
trout testis during spermatogenesis</u>

Testis homogenates, from trout injected twice weekly with

a salmon pituitary extract and maintained as described in

Materials and Methods, were assayed for cyclic nucleotide

phosphodiesterase activities, at 30°, in standard incubation

mixtures at pH 7.5, as described in Materials and Methods.

Cyclic nucleotide concentration was 1 mM.   EGTA (250 µM)

was included in all assays.   The activity units are pmol

cyclic nucleotide hydrolyzed/min/mg protein.   This graph

illustrates data from Table III.   ●—● cyclic AMP

  O—O cyclic GMP

B. <u>Cyclic nucleotide phosphodiesterase activities, measured
at micromolar substrate concentrations, in trout testis
during spermatogenesis</u>

Legend as above, except that cyclic nucleotide concentration

was 1 µM.   This graph illustrates data from Tables IV and V.

Symbols as above.
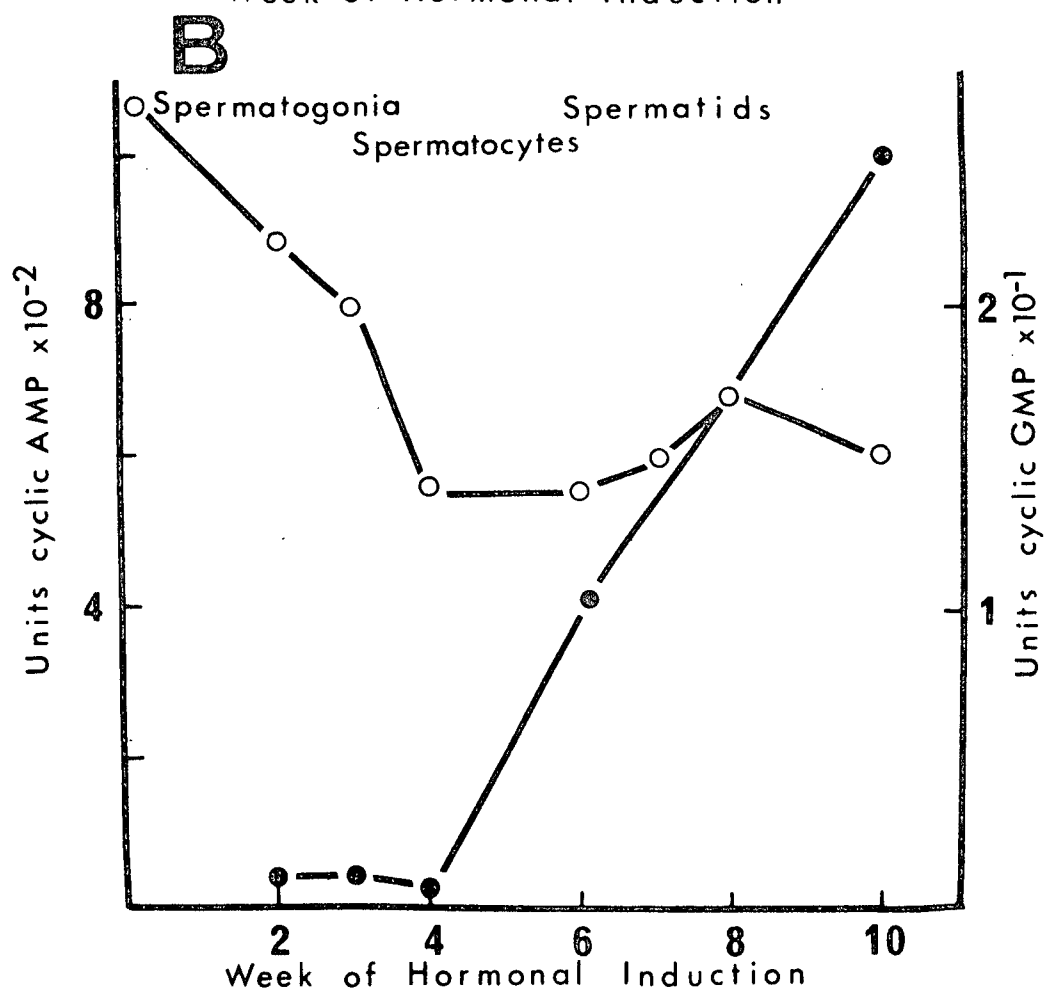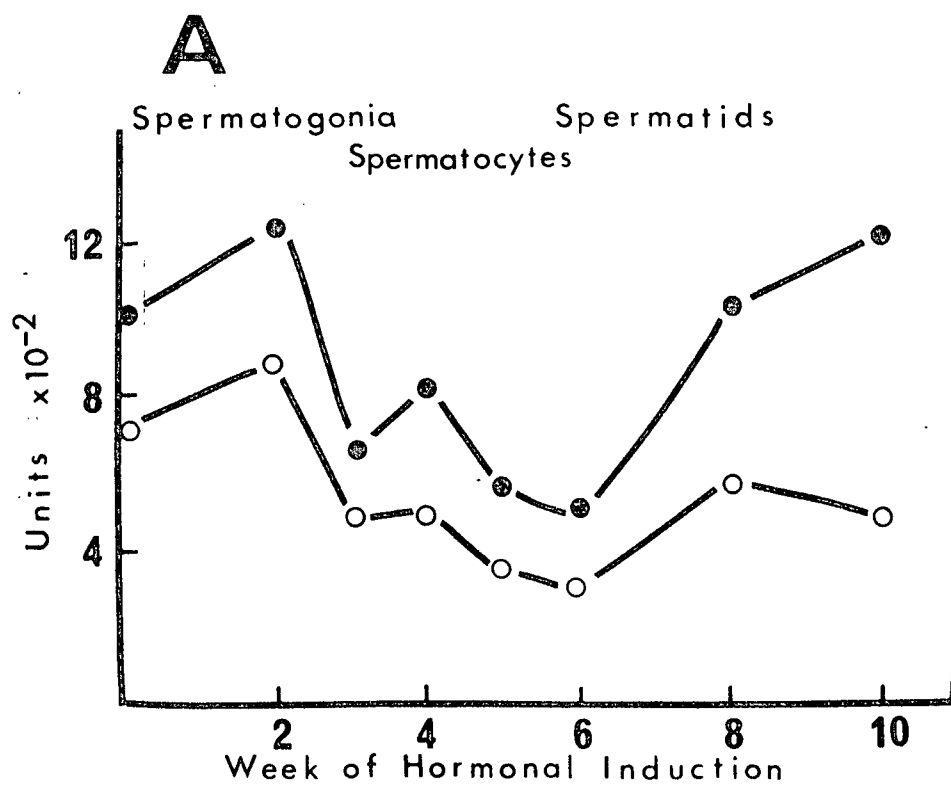
A

Spermatogonia   Spermatids
            Spermatocytes

Units ×10⁻²

Week of Hormonal Induction

B

Spermatogonia   Spermatids
            Spermatocytes

Units cyclic AMP ×10⁻²

Units cyclic GMP ×10⁻¹

Week of Hormonal Induction

TABLE V

CYCLIC GMP PHOSPHODIESTERASE ACTIVITIES MEASURED AT

MICROMOLAR SUBSTRATE IN TROUT TESTIS DURING HORMONALLY-

INDUCED SPERMATOGENESIS

Week of Hormonal Induction	Total Testes Wet Weight (mg)	Cyclic GMP Phosphodiesterase Activities		
		No Addition	Plus EGTA [a]	Plus Ca^{2+} [b]
0	30	43	27	45
2	80	27	24	29
3	410	29	23	27
3	1000	19	18	21
6	2500	14	12	13
7	9000	20	15	22
8	28000	18	17	19
10	18000	40	18	41

Legend as in Table III, except assays were measured at micromolar substrate concentrations,

[a] EGTA (250 µM) in assay mixture
[b] Ca^{2+} (100 µM) in assay mixture

two cases, zero time and completely mature testis, was a
marked inhibition by EGTA noted (30 and 55% inhibition,
respectively). In all other stages of development, there
was a 12-25% inhibition by EGTA. Activity in the presence
of Ca^{2+} was approximately the same as that observed with no
additions. There was no specific high affinity cyclic GMP
phosphodiesterase activity, in the presence or absence of
Ca^{2+}, associated with the premeiotic stage in which the large
decrease in cyclic GMP concentration was observed (Figure 4).

4. DEAE-cellulose chromatographic fractionation of cyclic AMP
   and cyclic GMP phosphodiesterases

When chromatographed on DEAE-cellulose, trout testis
homogenates, from all stages of development, yield two active
cyclic nucleotide phosphodiesterase fractions. These are
eluted by about 0.35 M (Peak I) and 0.65 M (Peak II) sodium
acetate, pH 6.5 (Figure 11). Column fractions were assayed
at low substrate concentrations ($\simeq$ 0.1 µM). One profile
(Figure 11B) was assayed in the presence of Ca^{2+} (100 µM),
in the presence of EGTA (250 µM) or with standard assay
incubation mixtures with no additions. No difference in
cyclic AMP or cyclic GMP phosphodiesterase activities were
observed under these different assay conditions.(data not
shown). This indicates the loss of Ca^{2+}-dependent phospho-
diesterase protein activator during DEAE-cellulose column
purification, as has been previously been reported (75).
Early attempts at fractionation of bovine heart activities,

FIGURE 11

DEAE-cellulose fractionation of cyclic AMP and cyclic GMP
phosphodiesterase activities in trout testis during
hormonally-induced spermatogenesis

DEAE-cellulose profiles of cyclic nucleotide phospho-
diesterases in trout testis homogenates prepared as
described in Materials and Methods.

A. Two weeks of hormonal induction. Average testes weight
146 mg; 280 mg in homogenate form applied to a column of
0.5 x 10.5 cm size. Column fraction size 2.2 ml.

B. Four weeks of hormonal induction. Average testes weight
629 mg; 540 mg in homogenate form applied to a column of
1 x 15 cm size. Column fraction size 4.5 ml.

C. Six weeks of hormonal induction. Average testes weight
3550 mg; 666 mg in homogenate form applied to a column of
0.8 x 22 cm size. Column fraction size 4.5 ml.

D. Eight weeks of hormonal induction. Average testes weight
4030 mg; 666 mg in homogenate form applied to a column of
0.8 x 22 cm size. Column fraction size 4.5 ml.

For all profiles, after sample application columns were
washed with 20 mM sodium acetate, pH 6.5 buffer containing
4 mM 2-mercaptoethanol. The initial wash contained no
phosphodiesterase activity. 20 mM - 1 M sodium acetate,
pH 6.5 linear gradients were started at tube 10 and cont-
inued through to tube 55, at a flow rate of 26 ml/hr.
Total gradient volume was 200 ml for profiles B, C and D.
Total gradient volume for A was 100 ml. Fraction 25 and
fraction 38 correspond to 0.35 and 0.65 M sodium acetate,
respectively.

Phosphodiesterase activities were assayed in 50 µl column
fraction aliquots (or 25 µl for profile A), at 30°, in
standard incubation mixtures, at pH 8.0, as described in
Materials and Methods. EGTA (250 µM) was in all assays.

●—● cyclic AMP (3 x 10^{-7}M; A and B. 1.5 x 10^{-7}M; C and D)

○—○ cyclic GMP (3 x 10^{-7}M; A and B. 1.6 x 10^{-7}M; C and D)
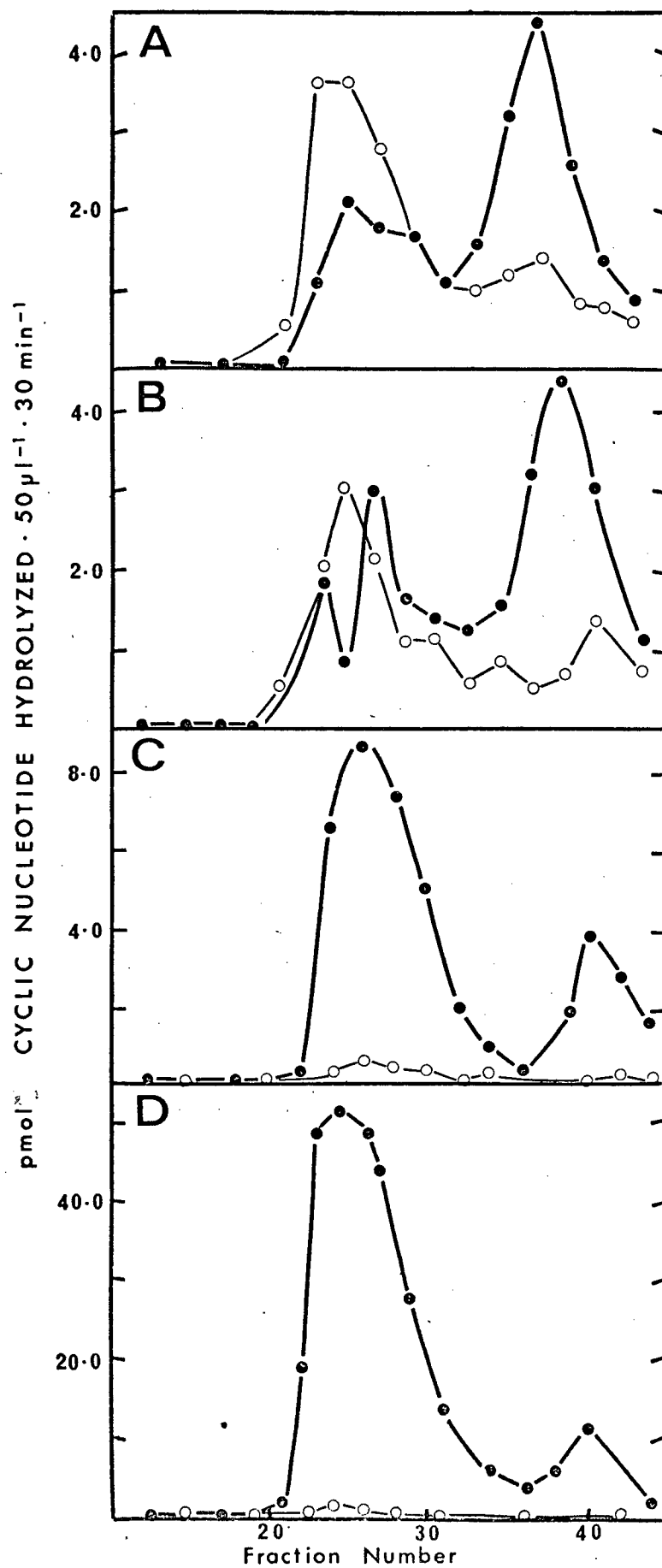
on DEAE-cellulose, were unsuccessful due to this loss (125).

At early stages of trout testis development (Figures 11A and 11B) an active cyclic GMP phosphodiesterase eluted at 0.35 M sodium acetate. This activity was unstable on prolonged storage at -20°, in contrast to the cyclic AMP activity which eluted at this position. The phosphodi- esterase activity eluting at 0.65 M sodium acetate was found to hydrolyze cyclic AMP almost exclusively.

In late stages of spermatogenesis (Figures 11C and 11D) a large increase in the relative size of Peak I to Peak II cyclic AMP phosphodiesterase activity occurs, while cyclic GMP hydrolysis is unchanged or slightly decreased. Quantitative comparisons between different stages of development cannot be made, due to the differing amounts of homogenates applied to columns and the lack of enzyme recovery data. Each fraction tube contained 4 mg of bovine serum albumin preventing the accurate measurement of enzyme protein. The 2 peaks at about 0.3-0.4 M sodium acetate, in Figure 11B, may be a partial separation of two enzyme forms, since this effect was also seen on a profile of testis homogenate from a trout after 3 weeks of hormone injections (profile not shown). However, the 100,000xg supernatant profile, from the same testis homogenate as in Figure 11B, did not show this double peak (Figure 13A).

A portion (33%) of Peak I, from the profile described in Figure 11D, was rechromatographed on DEAE-cellulose and only one peak, cochromatographing at the original Peak I

position, was observed (Figures 12A and 12B). In the rat
liver, it has been demonstrated that the low affinity cyclic
AMP phosphodiesterase peak from a Bio-Gel A-5m column, when
rechromatographed on DEAE-cellulose, gave a profile which
contained both the original low affinity activity, in a
0.3 M sodium acetate cut, plus a high affinity activity, in
a 0.6 M sodium acetate cut (60). No such Peak II activity
was observed in the present case (Figure 12B). There was
a 50% loss in cyclic AMP activity and about a 90% loss of
cyclic GMP activity, observed in Figure 11D, on rechromat-
ography of Peak I (Figure 12B). Cyclic GMP activity, in
the rechromatographed Peak I profile, was very low, and,
therefore was not included in Figure 12. The cause of the
activity losses is not known.

5. Soluble and particulate activities fractionated on
   DEAE-cellulose

When the 100,000xg supernatant (soluble) fraction,
from a trout testis homogenate, was chromatographed on
DEAE-cellulose, both cyclic AMP phosphodiesterase peaks
identified in the total homogenate profile, were observed
(Figure 13A). Cyclic GMP phosphodiesterase activity was
observed in the Peak I position, but was extremely low, and,
therefore was not included in Figure 13A. There was a 2.5
fold increase in Peak II to Peak I cyclic AMP activity ratio in
the soluble profile, as compared with the same ratio in the
homogenate profile (Figure 11B) from the same testis. A
sonicated 100,000xg pellet (particulate) fraction, from

FIGURE 12

A. DEAE-cellulose profile of cyclic AMP phosphodiesterases
in a testis homogenate from a trout injected with a salmon
pituitary extract for 8 weeks (detailed description in the
legend for Figure 11D). Phosphodiesterase activities were
assayed in 10 µl column fraction aliquots, at 30°, in
standard incubation mixtures, at pH 8.0, as described in
Materials and Methods. EGTA (250 µM) was in all assays.
Cyclic AMP concentration was 1.5 x 10^{-7}M.

B. DEAE-cellulose profile of rechromatographed peak I,
from the profile of the testis homogenate described above.
Peak I activity from the above profile was pooled, and
concentrated as described in Materials and Methods, and a
portion of the concentrate applied to a DEAE-cellulose
column. Conditions for DEAE-cellulose fractionation as
described in Materials and Methods. Phosphodiesterase
activities assayed as described above, except that cyclic
AMP concentration was 0.5 x 10^{-7}M.

pmol CYCLIC NUCLEOTIDE HYDROLYZED $\cdot$ 10 μl^{-1} $\cdot$ 30 min^{-1}

A

B

78.
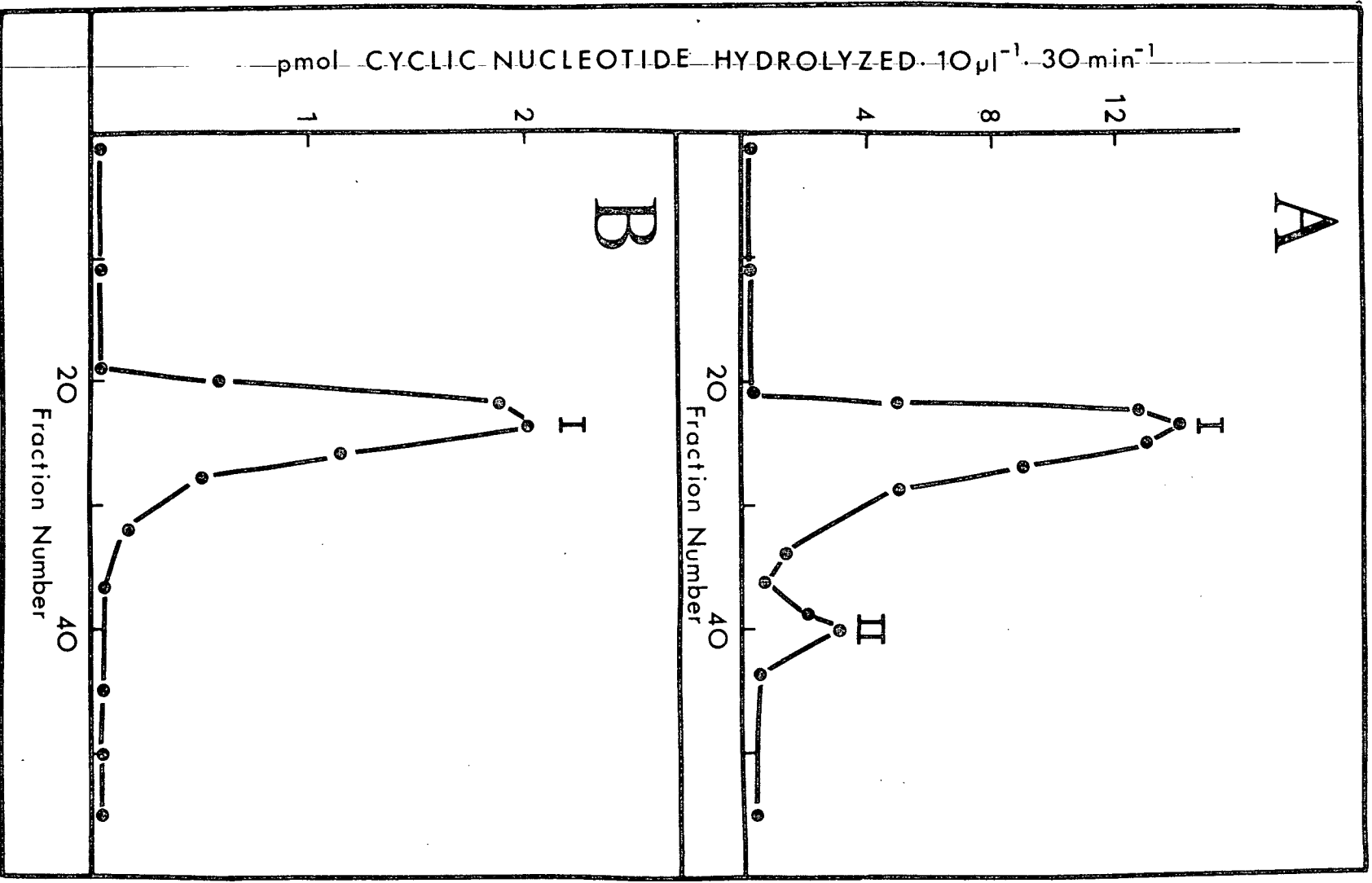
FIGURE 13

A. DEAE-cellulose profile of trout testis cyclic AMP

   phosphodiesterase activities in a 100,000xg supernatant

   fraction from testis hormonally-induced for 4 weeks.

   Average (from 2 fish) total testes weight of 629 mg.

B. DEAE-cellulose profile of trout testis cyclic AMP

   phosphodiesterase activities in a 100,000xg particulate

   fraction from the testis described above.

   The conditions for both separation profiles were as

described in detail in Materials and Methods.   The

gradient was from fraction 10 to fraction 50.   50 µl

column fraction aliquots were assayed for phosphodiesterase

activities, at 30°, in standard incubation mixtures at

pH 8.0, as described in Materials and Methods.   Cyclic AMP

assay concentration was 1.5 x 10^{-7}M.   EGTA (250 µM) was

included in assay mixtures.

pmol : CYCLIC NUCLEOTIDE HYDROLYZED · 50 ul⁻¹ · 30min⁻¹

80.
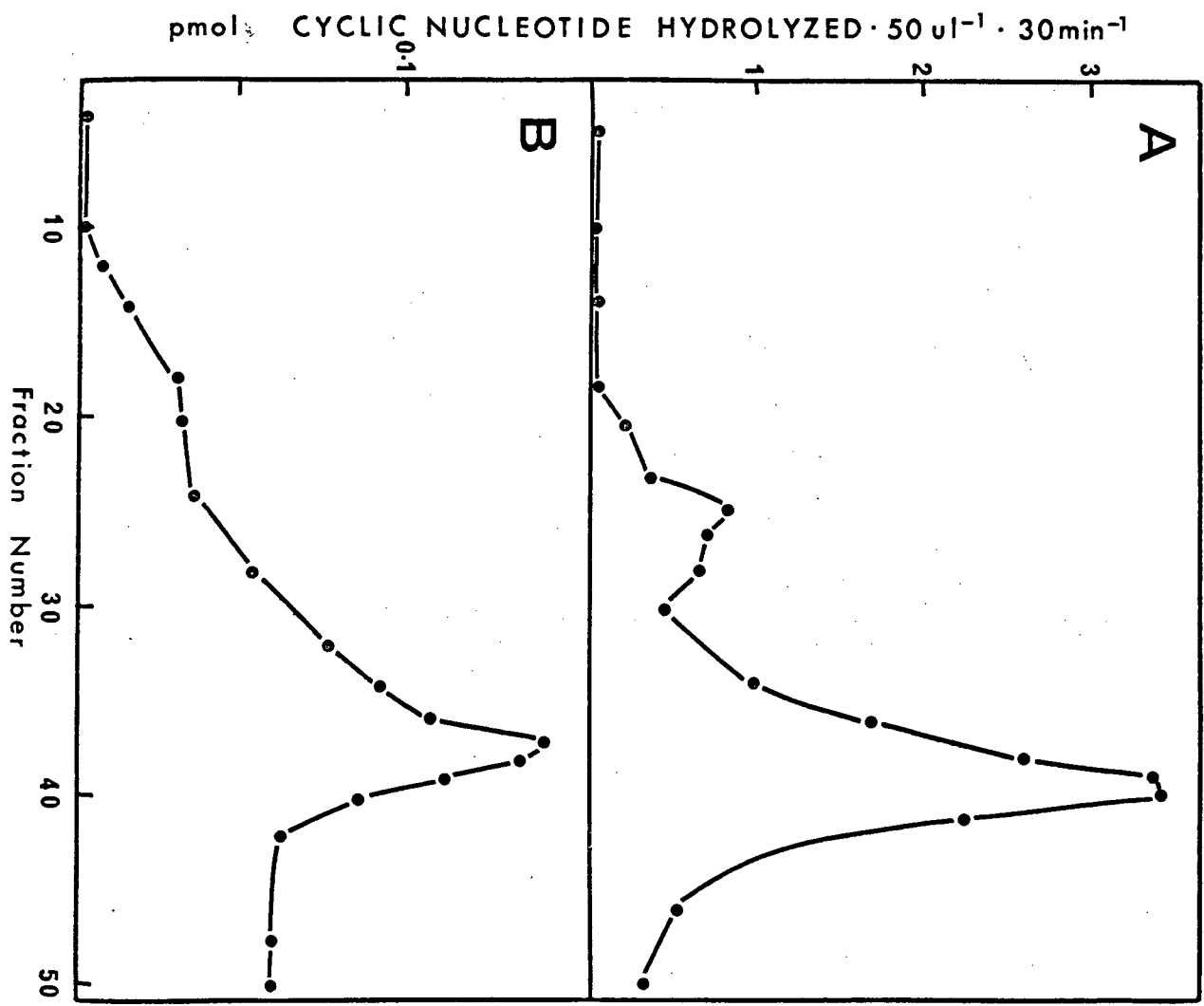
the same trout testis homogenate, contained mainly Peak II
cyclic AMP activity (Figure 13B).

The particulate fraction, from trout testis homogenate,
contained about 15% of the cyclic AMP phosphodiesterase
activities and 20% of the cyclic GMP phosphodiesterase
activities, measured at micromolar substrate concentrations.
The specific activity of the 100,000xg pellet was 21 pmol
cyclic AMP hydrolyzed/min/mg protein, about 50% of that of
the 100,000xg supernatant at 43 pmol cyclic AMP hydrolyzed/
min/mg protein. For cyclic GMP hydrolysis, the specific
activity of the 100,000xg pellet was 3.7 pmol cyclic GMP
hydrolyzed/min/mg protein, about 75% of that of the 100,000xg
supernatant at 4.8 pmol cyclic GMP hydrolyzed/min/mg protein.

6. Kinetic analyses of cyclic AMP phosphodiesterase activities fractionated on DEAE-cellulose

Kinetic analyses on the cyclic AMP phosphodiesterase
activities, in the peaks of activity obtained from DEAE-
cellulose, were made in order to investigate their substrate
affinities. Two types of data expression were used, i.e.
Lineweaver-Burk plots and Hofstee plots.

A Hofstee plot of Peak I cyclic AMP phosphodiesterase
activities, from a mature testis homogenate, revealed a low
apparent Km_1 obtained from the linear slope in Figure 14.
Hofstee plots of Peak II activities, from both premeiotic
and mature testis homogenates, indicated similar low
apparent Kms (Figures 15B and 16B). Peak I activity from

FIGURE 14

Hofstee plot of the rate of cyclic AMP hydrolysis by

DEAE-cellulose Peak I from testis from trout hormonally-

induced for 8 weeks (detailed description in the legend

for Figure 11D).   The unit of velocity is pmol/min/10 µl.

Assays were at 30°, in standard incubation mixtures at

pH 7.5, as described in Materials and Methods.   EGTA

(250 µM) was included in all assays.   Substrate conc-

entration ranged from 0.05 - 5 µM.

FIGURE 15

A. Lineweaver-Burk plot of the rate of cyclic AMP hydr-
olysis of DEAE-cellulose Peak II from testis from trout
hormonally-induced for 3 weeks (total testes weight 250 mg).
The unit of velocity is pmol/min/10 µl.   Assays were at 30°,
in standard incubation mixtures, at pH 7.5, as described in
Materials and Methods.   EGTA (250 µM) was included in all
assays.   Substrate concentration ranged from 0.05 - 5 µM.

B. Hofstee plot of the hydrolysis described above.

   The Lineweaver-Burk plot gives two apparent Kms of
0.8 µM and 2.3 µM.   The Hofstee plot gives two apparent Kms
of 0.5 µM and 4.4 µM.
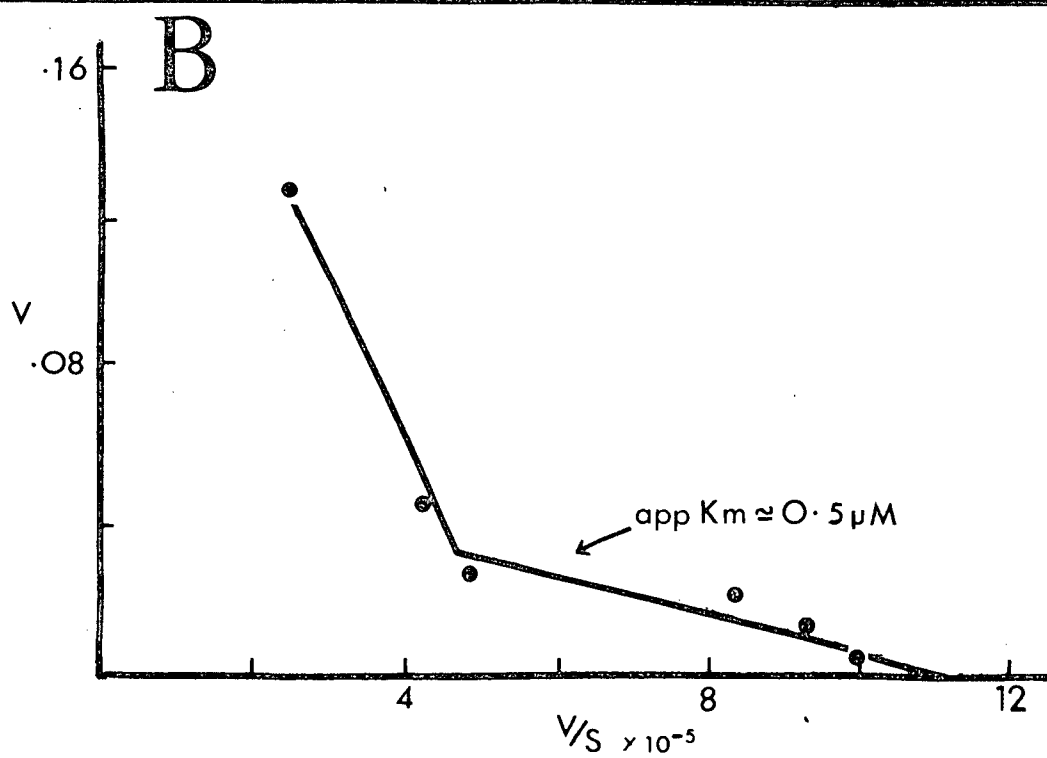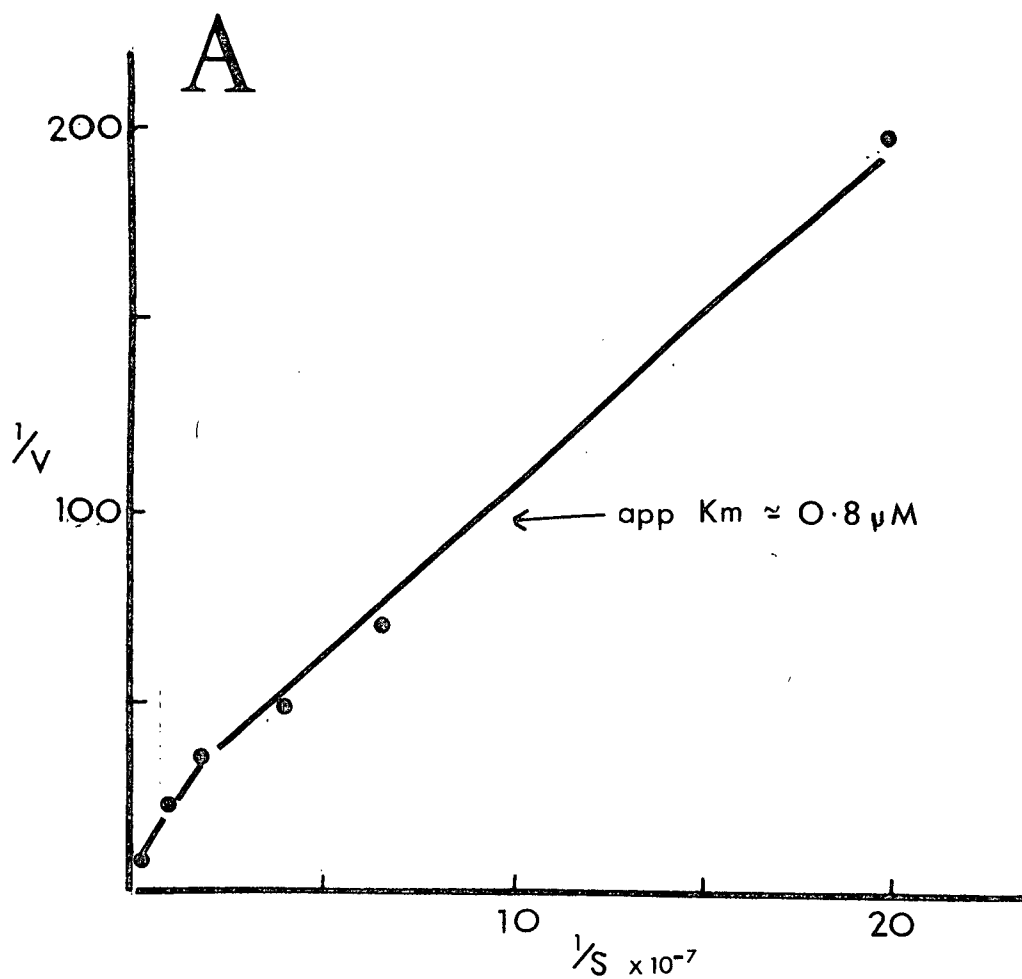
FIGURE 16

A. Lineweaver-Burk plot of the rate of cyclic AMP hydrolysis

by DEAE-cellulose Peak II from testis from trout hormonally-

induced for 8 weeks (detailed description in the legend for

Figure 11D).   The unit of velocity is pmol/min/10 µl.

Assays were at 30°, in standard incubation mixtures at pH 7.5

as described in Materials and Methods.   EGTA (250 µM) was

included in all assays.   Substrate concentration ranged

from 0.05 - 5 µM.
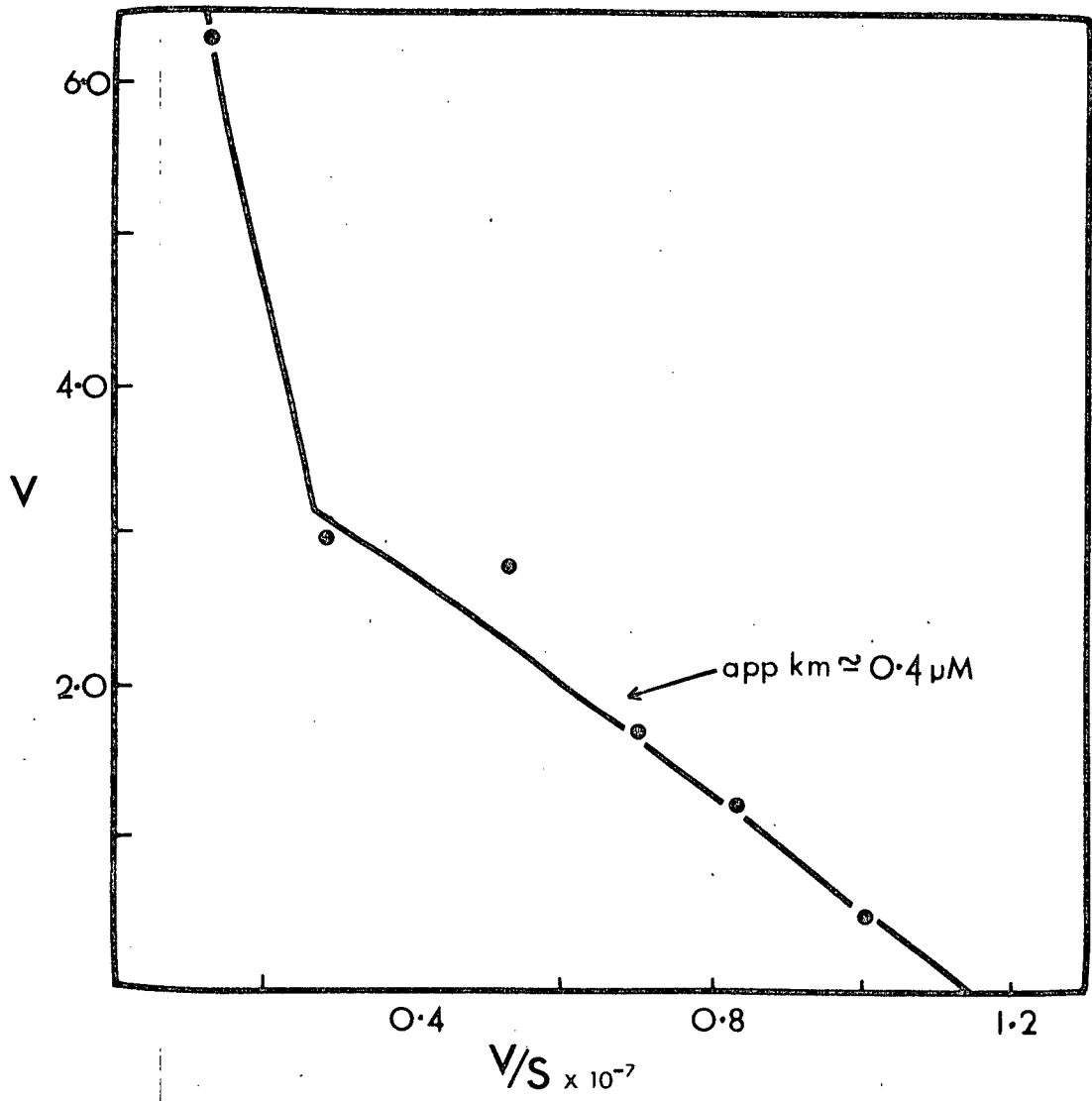

B. Hofstee plot of the hydrolysis described above.

    The Lineweaver-Burk plot gives a Km ≃ 1.0 µM.

The Hofstee plot gives an apparent Km of 0.7 µM.
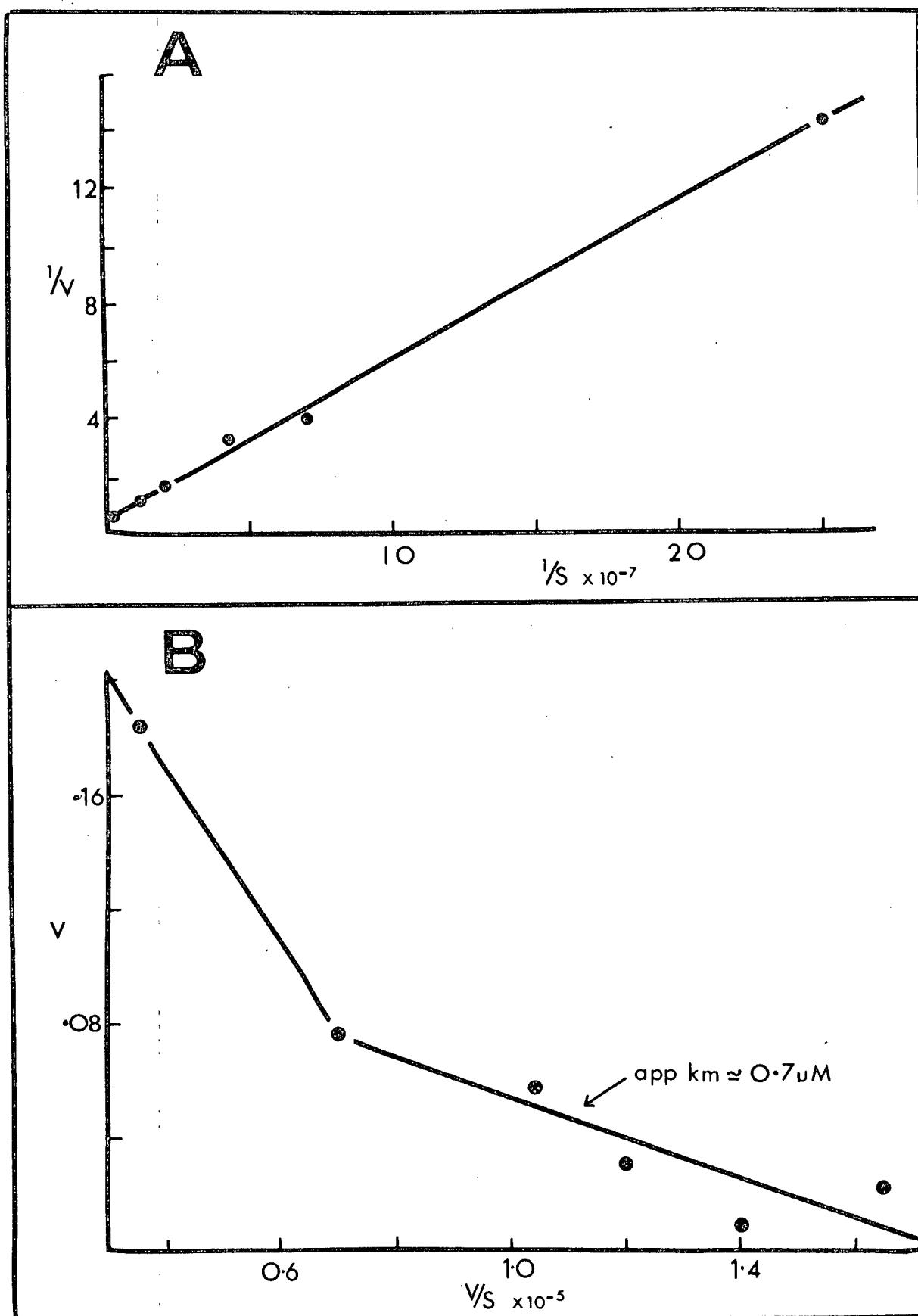
premeiotic testis homogenate was too low to obtain reliable
kinetic data from.

The non-linearity of both Lineweaver-Burk (Figure 15A)
and Hofstee (Figures 14, 15B and 16B) plots of cyclic AMP
phosphodiesterase activities is commonly observed in
kinetic studies on phosphodiesterase activities in various
tissues (60, 90, 133). Two apparent Km values could be
obtained from the two slopes on both the Lineweaver-Burk
plot and the Hofstee plot of cyclic AMP hydrolysis by
Peak II phosphodiesterase from the premeiotic testis homo-
genate (Figures 15A and 15B). The apparent Kms from all
plots corresponded to phosphodiesterase activities with
high affinity for cyclic AMP.

D. Guanylate cyclase activities in trout testis during

spermatogenesis

1. Assay system

In crude preparations, guanylate cyclase activities are

usually small compared to the activities of interfering

enzymes, such as nucleoside triphosphatases, cyclic nucleo-

tide phosphodiesterases, nucleotidases and deaminases (126).

Since the products, of the reactions which these contaminating

enzymes catalyze, may cochromatograph with cyclic GMP, an

effective method of separating cyclic GMP from GTP and

all its possible degradation products, is imperative (126).

Assays utilizing radioactive GTP provide the maximum

sensitivity. If $[\alpha^{32}P]GTP$ is used, separation of cyclic

GMP from all other purine nucleotides and inorganic phosphate,

is required. With $[^3H]$ or $[^{14}C]GTP$ as substrate, additional

separation from all purine nucleosides, bases and uric acid

is necessary. Methods for such separations are the same

as those mentioned for cyclic nucleotide purification. A

particularly popular method for assaying guanylate cyclase

activities uses $[\alpha^{32}P]GTP$ as substrate and purification

of reaction products on neutral alumina (127).

A modification of the latter method, has been developed

to provide greater sensitivity for the assay of guanylate

cyclase activities (106). The modified method utilizes a

combination of DOWEX 50 ion exchange chromatography and neutral

alumina adsorption chromatography, in the separation of

reaction products. This method was chosen for the present research, due to its low blank of $[\alpha^{32}P]GTP$ in the purified $[^{32}P]$cyclic GMP fraction, its ease of performance, its reliability and for the previous rigorous identification of the $[^{32}P]$ product as cyclic GMP (106).

Assay conditions and incubation mixtures closely followed those described in the paper which detailed the two-stage column procedure (106). In this paper accurate and reliable guanylate cyclase assays were carried out in a variety of tissues. The assay temperature of 37° was used, because guanylate cyclase activities in fish sperm (88) had been carried out at 37°.

In the present research, the incubation contents outlined previously (106) were modified to include a cyclic nucleotide phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine, and a GTP regenerating system, consisting of 15 mM phosphocreatine and 12.25 units of creatine phosphokinase per assay tube. The GTP regenerating system was found to be an absolute requirement for the detection of the total guanylate cyclase activities in trout testis homogenates. Without the GTP regenerating system only 4% of the guanylate cyclase activity present was detected. The modified incubation mixture also contained 2-mercaptoethanol to prevent spontaneous air oxidation of the soluble guanylate cyclase, as has been reported in other tissues (70).

2. Activities and properties during testis development

Guanylate cyclase activities were measured in the
100,000xg pellet (particulate) fraction and in the 100,000xg
supernatant (soluble) fraction, of trout testis homogenates
from zero time trout, and from trout after 3, 6, and 10 weeks
of twice weekly salmon pituitary extract injections (Tables VI
and VII). The particulate and soluble specific activities
decreased 6 fold and 4 fold, respectively, during the 10
weeks of testicular maturation (Table VI). Both total part-
iculate and soluble activities decreased 4 fold over the same
period (Table VII). The majority of the decreases in both
specific and total activities, occurred between the third
and sixth week of hormone injections. During this time,
there was a 2 fold decrease in particulate specific activity
and a 3 fold decrease in soluble specific activity (Table VI),
and an approximate 3 fold decrease in both total particulate
and soluble activities (Table VII). Not enough testes were
sampled in the present guanylate cyclase study to correlate
the enzyme decreases with a precise stage of spermatogenesis.

A guanylate cyclase particulate to soluble ratio of 1.9
was observed for specific and total activities in zero time
testis and in fully mature testis (Tables VI and VII). The
total particulate guanylate cyclase activity appeared to
increase prior to meiosis (week 3 of hormonal induction;
Table VII), resulting in a particulate to soluble ratio of
2.9 at this stage of development.

Triton X-100, a non-ionic detergent, has been

TABLE VI

GUANYLATE CYCLASE SPECIFIC ACTIVITIES IN TROUT TESTIS

DURING HORMONALLY-INDUCED SPERMATOGENESIS

Week of Hormonal Induction	Total Testes Wet Weight (mg)	Guanylate Cyclase Activities	
		100,000xg Supernatant	100,000xg Pellet
0	130	14.8 ± 0.9	27.9 ± 2.0
3	350	11.7 ± 0.8	21.8 ± 2.4
6	3300	4.3 ± 0.4	9.7 ± 0.7
10	22000	3.9 ± 0.7	4.7 ± 0.2

Crude homogenates of trout testis were prepared in buffer A and 100,000xg supernatant and pellet fractions obtained, as described in Materials and Methods. Activity assays were at 37°, in standard incubation mixtures at pH 7.5, as described in Materials and Methods. Activity units are pmol cyclic GMP formed/min/mg protein. Values are the average of duplicate assays on the same preparation. The $\pm$ value indicates the range between assay duplicates.

TABLE VII

TOTAL GUANYLATE CYCLASE ACTIVITIES IN TROUT TESTIS

DURING HORMONALLY-INDUCED SPERMATOGENESIS

Week of Hormonal Induction	Total Guanylate Cyclase Activities		
	100,000xg Supernatant	100,000xg Pellet	Total[a]
0	0.38	0.70	1.08
3	0.37	1.04	1.41
6	0.12	0.40	0.52
10	0.09	0.19	0.28

Legend as for Table VI, except that activity units are pmol cyclic GMP formed/min/mg testis wet weight.

[a]Total obtained by the summation of the 100,000xg pellet and supernatant activities. Total homogenate activity was not assayed.

shown to activate guanylate cyclase activities in other

tissues (64), specifically in the particulate fractions. It

was investigated with regard to its effect on trout testis

guanylate cyclase activities during spermatogenesis

(Figure 17A).   Triton X-100 (1% concentration) stimulated

particulate guanylate cyclase activities, at all stages of

trout testis development, approximately 2 fold (Figure 17A).

The soluble guanylate cyclase activity, in zero time testis,

was also stimulated by Triton X-100 (Figure 17B).   The

latter stimulation was 1.8 fold, compared with 2.2 fold

stimulation, by Triton X-100, of particulate guanylate

cyclase activity in zero time testis.

The effect of a gonadotropin extract on guanylate cyclase

activities, in zero time testis, was also investigated

(Figure 17B).   (The gonadotropin extract used was the standard

salmon pituitary preparation used in the initiation and

enhancement of trout spermatogenesis, as described in

Materials and Methods.)   The gonadotropin extract caused

an apparent 10 fold stimulation of the soluble zero time

activity and an apparent 2 fold stimulation of the particulate

zero time activity.   Addition of both Triton X-100 and

gonadotropin extract, to the particulate fraction resulted

in a slightly less than additive increase i.e. 3.2 fold

stimulation (Figure 17B).   No stimulation of activity

occurred with the addition of heat-denatured gonadotropin

extract (data not shown).   However, when the gonadotropin

extract was assayed without any testis protein present, a

FIGURE 17

A. Triton X-100 effect on guanylate cyclase activity in the 100,000xg pellet from testis of zero time trout (OP), and from testis of trout after 3 (3P), 6 (6P), and 10 (10P) weeks of twice weekly hormone injections, as described in Materials and Methods.   Total testes weights and tissue preparation as described in the legend for Table VI. Activities were assayed at 37°, in standard incubation mixtures, as described in Materials and Methods.   Error bars indicate the range between assay duplicates.

☐   No additions

▨   Plus 1% Triton X-100 (x)

B. Comparison of the effects of salmon gonadotropin and Triton X-100 on the 100,000xg pellet (OP) and the 100,000xg supernatant (OS) zero time trout testis guanylate cyclase activities.   Activities were assayed as described above. Symbols as above, plus:

◨   Plus salmon gonadotropin (h)
      (15 μl crude pituitary extract)

▨   Triton X-100 (1%) and salmon
      gonadotropin (hx)

pmol cyclic GMP formed·min⁻¹· mg protein⁻¹
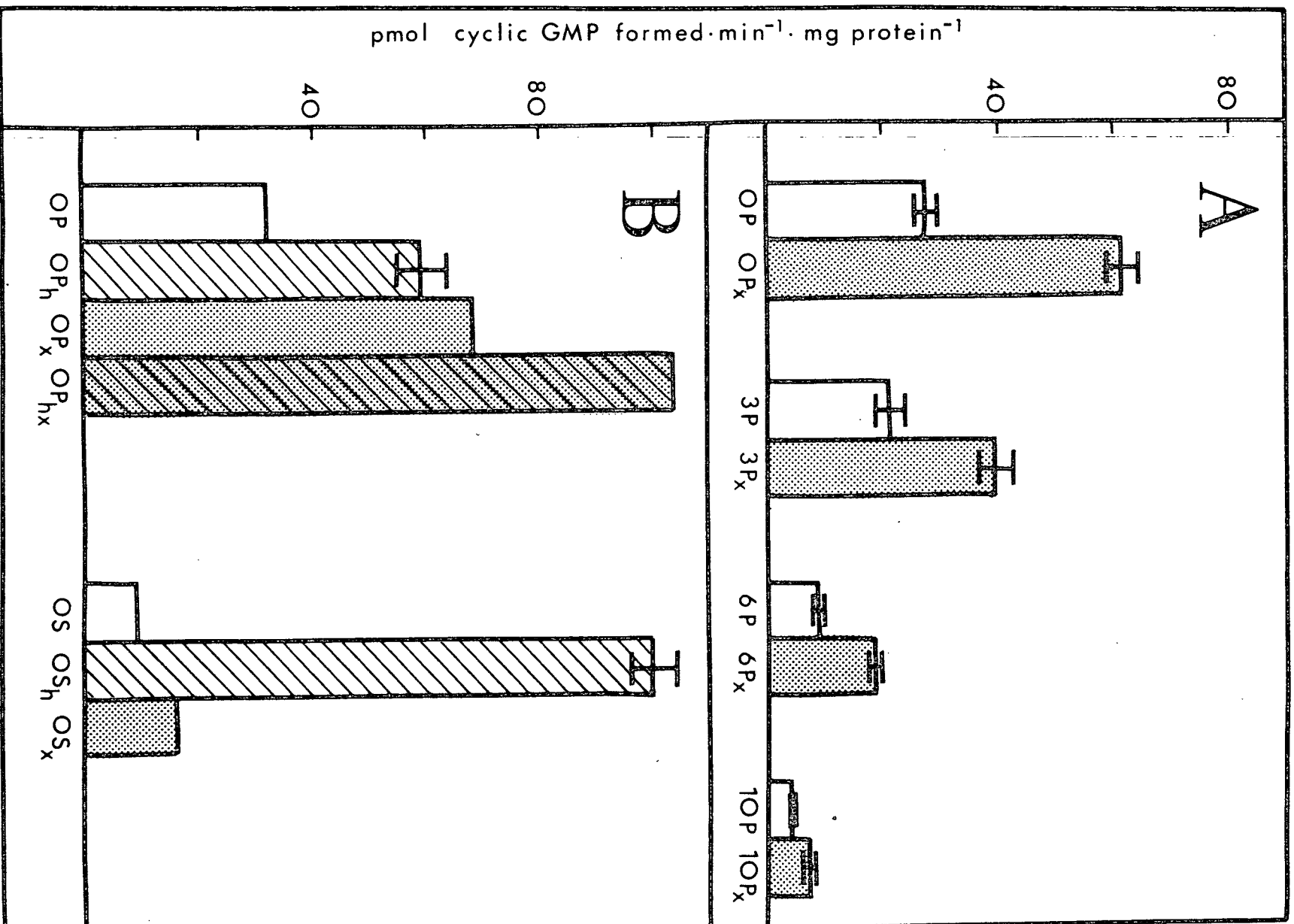
considerable endogenous guanylate cyclase activity was observed; 4.23 pmol/min/15 μl of gonadotropin extract, or 11.3 pmol/min/mg gonadotropin extract protein.   Since the unstimulated soluble guanylate cyclase activity was lower than the unstimulated particulate activity, the same addition of gonadotropin extract to each, produced a greater apparent stimulation of the soluble activity.   Both increases in activity are totally attributable to the endogenous guanylate cyclase activity in the gonadotropin extract.

DISCUSSION

Cyclic GMP and cyclic AMP concentrations in trout testis during spermatogenesis

Cyclic GMP concentrations in immature trout testes were revealed to be high (about 2 µmol/kg testis wet weight) and equal to cyclic AMP concentrations in immature trout testes. In most vertebrate tissues cyclic GMP concentrations are in the range of 0.05-0.1 µmol/kg wet weight (53) and cyclic AMP concentrations are about 10-50 fold higher (64). In several tissues, including lung, cerebellum and lymph, cyclic GMP and cyclic AMP concentrations are approximately equal and in the normal cyclic AMP concentration range (53, 64). In one vertebrate tissue, namely the retina, extremely high cyclic GMP concentrations are observed (about 100 µmol/kg wet weight) and there is a cyclic GMP to cyclic AMP ratio of 100 (141). In one invertebrate tissue, namely the male cricket reproductive accessory gland, very high cyclic GMP concentrations are also found, 40-100 µmol/kg wet weight, while cyclic AMP concentrations are only 0.02-0.3 µmol/kg wet weight (130). The high cyclic GMP concentrations in the latter case were shown to be associated with the gland itself and not the sperm it contains, since high cyclic GMP was also observed in the reproductive glands from castrated crickets (130).

In immature trout testis it has been estimated that spermatogonial germ cells make up about 10-15% of the total wet weight and the remainder is due to connective tissues and the cells within this component such as Leydig cell homologues and blood and nerve cells (10). One cannot conclude which of these cell types may have an especially high

concentration of one cyclic nucleotide , cyclic GMP or cyclic AMP, or both. However, over a period of a 5-10 fold increase in testicular wet weight, during which time the germ cell component would be increasing rapidly, cyclic GMP concentrations remained high (Figure 4). Therefore, it is quite possible that elevated cyclic GMP concentrations are characteristic of spermatogonia, as well as perhaps other cell types present in the immature trout testis.

Several lines of research have suggested that cyclic GMP may act as a positive signal (7, 8) while cyclic AMP may act as a negative signal (131, 132) in the cell for controlling the growth of cultured fibroblasts. Reciprocal concentrations of cyclic GMP and cyclic AMP have been observed during the cell cycle of Novikoff hepatoma cells (95). Elevated cyclic GMP during mitosis and S phase were considered to be consistent with potential modulatory roles for cyclic GMP in proliferation (95). There was no evidence for reciprocal cyclic GMP and cyclic AMP concentrations in trout testis during development. In fact in immature testis the concentrations were equal. However, the heterogeneity of cell types present in immature trout testis prevents the observation of relative cyclic nucleotide concentrations in specific cell types. Goldberg states in the Yin Yang hypothesis of cyclic nucleotide regulation, that the relative proportions of cellular cyclic GMP and cyclic AMP may be more important than their actual concentrations (53). The Yin Yang hypothesis, based on the ancient oriental concept of a dualism between opposing natural forces which may enter into

a mutual interaction that results in synthesis, was used to explain the strikingly antagonistic regulatory influences of cyclic GMP and cyclic AMP in several biological systems. (53, 133, 134). In more recent research (54) the roles of both cyclic GMP and cyclic AMP in regulation in various cell types has become a subject of considerable controversy. There is growing evidence that the cyclic nucleotides do not act alone as modulatory effectors but may act in concert with modulators such as Ca^{2+} and lipids to promote or inhibit a cellular event (54).

The most striking aspect of the present study of cyclic nucleotides in developing trout testis is the sharp (during 1 week of the 12 week process) 10 fold decrease in cyclic GMP after about 4 weeks. (Figure 4). This decrease occurs at the middle of the logarithmic growth of the trout testis (Figure 3) shortly before the onset of meiosis at weeks 5 to 6 (10). Although there is uncertainty about the exact timing of meiosis in the present study, it is obvious from Figure 4 that the decrease in cyclic GMP occurred before the proliferation of germ cells had ceased. The magnitude and timing of the decrease in cyclic GMP suggest that it may be critical for the onset of meiosis. To substantiate this hypothesis it would be important to isolate specific cell types rapidly to determine whether spermatogonia have significantly greater concentrations of cyclic GMP than primary spermatocytes.

In rat testis, the binding of immunofluorescent antibodies to cyclic GMP to prophase chromosomes in primary spermatocytes(79) is consistent with an important role for cyclic GMP at meiosis.

After the sharp 10 fold decrease in cyclic GMP in trout
testis at the onset of meiosis, cyclic GMP decreased gradu-
ally another 5 fold during the remainder of spermatogenesis
(Figure 4).   After the 2 fold decrease in cyclic AMP in
trout testis prior to meiosis (Figure 5), cyclic AMP did not
change significantly during the following stages of meiotic
reduction and spermatid differentiation.

The cyclic nucleotide concentrations in developing trout
testis can be compared with those in rat testis during matur-
ation (79).   Elevated initial concentrations of both cyclic
GMP and cyclic AMP were also observed in the rat testis, but
the initial cyclic AMP concentration was 40 fold higher than
the initial cyclic GMP concentration.   Both cyclic nucleo-
tides decreased about 2 fold at the time of the first reduc-
tive divisions in rat testis.   The particularly elevated
cyclic GMP concentrations observed in trout testis during
early development and the striking decrease in these concent-
rations at the onset of meiosis appear to be special to this
system.   The large multiplication of spermatogonial germ
cells and the synchrony of germ cell development in the trout
testis may contibute to these observations.   During the
remainder of rat testicular development, fluctuations in cyclic
GMP were seen, but there was an overall decrease in concent-
ration.   There was a larger and clear trend of decrease in
cyclic GMP in trout testis during spermatid differentiation,
which may result from the homogeneity of germ cell type
present in trout testis.   There is much greater germ

cell heterogeneity in rat testis.(1, 10). Cyclic AMP
decreased steadily in rat testis until the time of the
appearance of spermatids (79). During spermatid differ-
entiation the cyclic AMP concentration increased to about
half of the immature rat testis concentration. Cyclic
AMP did not increase in trout testis during spermatid
differentiation, suggesting that this cyclic nucleotide
may play a more important role in spermatids of mammals
than of fish.

It would be desirable to combine cyclic nucleotide de-
terminations in trout testis , with immunofluorescent cyclic
nucleotide binding studies and cell separation methods, to
determine the predominant sites of the cyclic nucleotides
and their receptor proteins.

The observation of the large and abrupt decrease in
cyclic GMP early in the development of trout testis, init-
iated an investigation into some of the factors which may
be important in the control of cellular cyclic GMP concent-
rations. In particular cyclic GMP phosphodiesterase activ-
ities and guanylate cyclase activities in developing trout
testis were studied. Extrusion of cyclic nucleotides is
another potentially important method of controlling cyclic
nucleotide concentrations in trout testis, but this was not
investigated in the present research.

Cyclic nucleotide phosphodiesterase activities in trout

testis during spermatogenesis

The fact that the total cyclic GMP phosphodiesterase activity in trout testis did not change significantly throughout spermatogenesis (Table III) indicated no obvious relation between cyclic GMP phosphodiesterase activity and cyclic GMP concentration. This conclusion was confirmed by the studies on cyclic GMP phosphodiesterases measured with micromolar substrate concentration during development (Tables IV and V) and the profiles of phosphodiesterase activity on DEAE-cellulose (Figure 11). These studies showed that there was no induction of a high affinity cyclic GMP phosphodiesterase at the time of the large decrease in cyclic GMP concentration.

EGTA inhibited total cyclic AMP and cyclic GMP phosphodiesterase activities in trout testis from 10 to 50% at different stages of development, except for a 30% stimulation of cyclic GMP phosphodiesterases in immature testis (Table III). The inhibition by EGTA indicates the presence of an active Ca^{2+}-binding activator of cyclic nucleotide phosphodiesterase activities in trout testis. A Ca^{2+}-binding protein capable of activating cyclic nucleotide phosphodiesterases has been isolated from sea urchin sperm (133) but this protein activator had no effect on endogenous sea urchin sperm cyclic nucleotide phosphodiesterases isolated on DEAE-cellulose. It is apparent in several tissues that the Ca^{2+}-binding protein may be involved with other metabolic functions, such as

protein phosphorylation (134), besides phosphodiesterase activation. The possibility of the induction of a Ca^{2+}-dependent high affinity cyclic GMP phosphodiesterase activity associated with the large decrease in cyclic GMP in developing trout testis was investigated (Table V). No change in the Ca^{2+}-dependent cyclic GMP phosphodiesterase activity was found at the onset of meiosis.

Both cyclic AMP and cyclic GMP phosphodiesterase activities measured at micromolar substrate concentrations were higher in immature trout testis than in testis at the spermatocyte stage of development (Figure 10B). In a study of changes in cyclic AMP and cyclic GMP phosphodiesterase activites in rat liver and lung tissues, higher activities were found in fetal than in neonatal tissues, and the activities in neonatal tissues were in turn higher than those in adult tissues (135). A reverse pattern was observed in developing brain (135). In all these tissues the magnitude of cyclic GMP phosphodiesterase activities at different stages of development, correlated well with the magnitude of cyclic GMP protein kinase activities (136). On the basis of high cyclic GMP phosphodiesterase and protein kinase activities in fetal lung and adult brain, it was hypothesized that actions modulated by cyclic GMP were important in these tissues (135). An investigation of cyclic GMP protein kinase activities in trout testis cells during development might reveal the site of action of cyclic GMP at the onset of meiosis.

The most striking change in cyclic nucleotide phosphodiesterase activities in developing trout testis is the induction of a cyclic AMP phosphodiesterase with high substrate affinity, which appears at about week 5 of spermatogenesis (Table IV and Figures 10B, 11C and 11D). A high affinity cyclic AMP phosphodiesterase activity associated with the onset of maturity has been found in rat and rabbit testis (93) and in ram sperm (94).

DEAE-cellulose chromatography of trout testis homogenates at different stages of development illustrated two peaks of cyclic AMP phosphodiesterase activity, the first of which also hydrolysed cyclic GMP (Figure 11). During spermatid development the induced high affinty cyclic AMP phosphodiesterase activity was observed to coelute with the first of the cyclic AMP peaks (Figures 11C and 11D). It is notable that there was no co-induction of a high affinity cyclic GMP phosphodiesterase activity. Kinetic analyses of DEAE-cellulose peak cyclic AMP phosphodiesterase activities from mature trout testis homogenate (Figure 11D) confirmed the presence of high affinity activities in both peaks (Figures 15 and 16). The non-linearity of these kinetic plots may result from multiple enzyme species in one peak, from negative co-operativity of a single enzyme species, or from interconvertible forms of phosphodiesterase with differing activities (60, 90).

Although no low affinity cyclic AMP phosphodiesterase was observed in mature trout testis (Figure 8), such an

activity is obviously present in immature testis, where
cyclic AMP phosphodiesterase activities were at least 40
fold higher when measured with millimolar cyclic AMP than
with micromolar substrate (Tables III and IV). There may
be little or no low affinity cyclic AMP phosphodiesterase
activity in mature testis, where activities measured with
millimolar cyclic AMP were only about 20% higher than those
measured with micromolar substrate (Tables III and IV).
This indicates that the induced high affinity cyclic AMP
phosphodiesterase is the predominant cyclic AMP phosphodi-
esterase activity present in mature testis.

Both a low and high affinity cyclic GMP phosphodiesterase
activity were observed in mature trout testis (Figure 9).
Cyclic GMP phosphodiesterase activities throughout develop-
ment were about 40 fold higher when measured with millimolar
cyclic GMP, than when measured with micromolar cyclic GMP
(Tables III and IV). This indicates that the low affinity
phosphodiesterase activity is predominant throughout sperm-
atogenesis.

Soluble cyclic AMP and cyclic GMP phosphodiesterase
activities, i.e. those found in the 100,000xg supernatant
fraction, when measured at micromolar substrate concentrat-
ions were about 85% and 80%, respectively, of similarly
measured total homogenate activities. A soluble fraction
from trout testis homogenate contained both peaks of cyclic
nucleotide phosphodiesterase activity eluted from DEAE-
cellulose (Figure 13A). The 2.5 fold increase in the ratio

of Peak II to Peak I cyclic AMP activity in the soluble
profile as compared with the same ratio in the total homog-
enate profile from the same testis (Figure 11B), may reflect
partial conversion of Peak I to Peak II on supernatant prep-
aration or storage at -20°.   Such an increase in the ratio
of Peak II to Peak I cyclic AMP phosphodiesterase activity
on DEAE-cellulose profiles has been observed in rat liver
preparations after storage of homogenates at 4° or after
mild treatment with trypsin (60).

The small amount of particulate cyclic AMP phosphodi-
esterase activity in the 100,000xg pellet fraction from
trout testis homogenate, contained mainly activity in the
second peak eluted from DEAE-cellulose (Figure 13B).   In
fractionation studies of rat liver phosphodiesterases (60)
the second cyclic AMP phosphodiesterase peak on DEAE-
cellulose profiles was isolated exclusively from the
100,000xg pellet fraction.

DEAE-cellulose profiles of male cricket reproductive
accessory gland homogenates were similar to those observed
in the present study on trout testis cyclic nucleotide
phosphodiesterases, i.e. a first peak hydrolyzing both
cyclic AMP and cyclic GMP and a second peak specific for
cyclic AMP (137).   The observation of no phosphodiesterase
with distinct specificity for cyclic GMP, in the trout testis,
even when cyclic GMP concentrations were high is also
observed in the reproductive accessory gland of male crickets.
No appreciable changes in the specific activity or kinetic

properties of accessory gland cyclic GMP phosphodiesterases were seen during a developmental period over which cyclic GMP concentrations rose more than 500 fold (137).

The cyclic nucleotide phosphodiesterase studies in trout testis do not indicate a selective role for phosphodiesterases in modulating cyclic GMP concentrations during spermatogenesis.

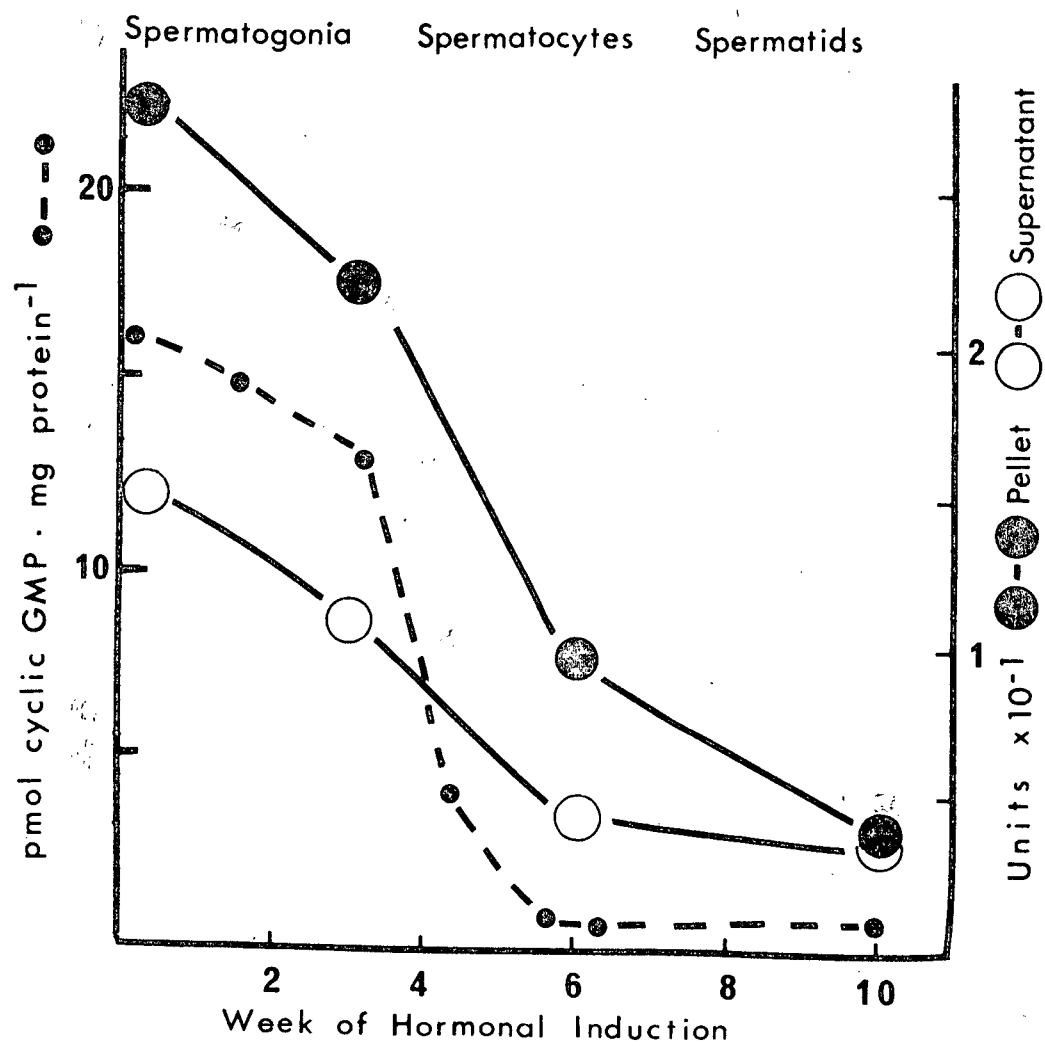Guanylate cyclase activities in trout testis during spermatogenesis

Guanylate cyclase activities in trout testis were invest-igated to determine their role in regulating cyclic GMP conc-entrations during spermatogenesis.   A direct correlation was found between guanylate cyclase activities and cyclic GMP con-centrations in developing trout testis, as is shown in Figure 18.   Surveys of guanylate cyclase activities in rat tissues have demonstrated a correlation between guanylate cyclase activity and cyclic GMP concentration (82, 138, 139).

In immature and mature trout testis, a particulate to soluble guanylate cyclase activity ratio of 1.9 was observed (Tables VI and VII).   A similar particulate to soluble ratio was observed in a study of guanylate cyclase activities in immature and adult rat testis (82).   Both particulate and soluble guanylate cyclase specific activities decreased during trout testis maturation (Table VI), but the partic-ulate enzyme showed a total activity increase just prior to meiosis (Table VII).   This could be related to a specific role for cyclic GMP in the nucleus at meiosis.   However, a more thorough investigation of guanylate cyclase activities during spermatogenesis is needed to substantiate this idea.

FIGURE 18

Comparison of guanylate cyclase activities and cyclic

GMP concentrations in trout testis during hormonally-

induced spermatogenesis.

Guanylate cyclase was assayed, in trout testis at differ-

ent stages of development, as described in Materials and

Methods.   Data from Table VI.   Units of activity are

pmol cyclic GMP formed/min/mg protein.   Cyclic GMP con-

centrations assayed, in trout testis at different stages

of development, as described in Materials and Methods.

Data from Table IA.

Triton X-100 (a non-ionic detergent) activated particulate guanylate cyclase activities in trout testis, at all stages of development, about 2 fold. This indicates that the particulate enzyme had the same membrane environment throughout spermatogenesis. Soluble guanylate cyclase activity in immature trout testis was activated by Triton X-100 to the same extent as the particulate enzyme. This suggests that there may be a close relation in form between the soluble and particulate enzyme. It has been suggested by Goldberg and others that the soluble form of guanylate cyclase may originate from cell membranes (54). Discussion on the importance of the two forms of guanylate cyclase, in mammalian tissues, has stressed the possible physiological regulation of the two forms (140). Increased particulate and decreased soluble guanylate cyclase activities have been found in regenerating rat liver, fetal rat liver, and hepatomas (140). These observations led to the speculation that particulate guanylate cyclase activity may be associated with tissue growth and that soluble activity may be associated with acquired functions of differentiation. The relatively constant ratio of particulate to soluble guanylate cyclase activity during trout testis development does not support this hypothesis.

No direct effect of a hormonal or physiological agent on guanylate cyclase activity has been convincingly demonstrated. The finding of an apparent stimulation of both particulate and soluble guanylate cyclase activities in immature trout

testis, by a salmon pituitary gonadotropin extract (Figure 16), was shown to be due to endogenous activity in the hormone preparation. The soluble testis guanylate cyclase, presumably not the physiological form which would respond to gonadotropin stimulation, was apparently stimulated to a greater extent than the particulate enzyme. Hormone stimulation of guanylate cyclases in other tissues has been reported to be suspect due to the impurities of the preparations used (54). In these cases also, the soluble guanylate cyclase was apparently stimulated to a greater extent than the particulate enzyme.

Conclusions

This study of trout spermatogenesis has revealed that during testis development there are three striking and precisely timed changes related to cyclic nucleotide metabolism. These are a decrease in cyclic GMP concentration, a decrease in guanylate cyclase activities and the induction of a high affinity cyclic AMP phosphodiesterase.

It is reasonable to assume that there is a connection between the decrease in cyclic GMP concentration, the decrease in guanylate cyclase activity and the onset of meiosis (Figure 18). Clearly further studies should be directed towards the identification of the cells in which the decrease in cyclic GMP concentration occurs and an investigation of the guanylate cyclase activities in those cells. If these experiments confirm the presumed

relationship, then it will be of great interest to define the nature of the decreased activity of the guanylate cyclases, and the source and nature of the signal which induces the change. Also of importance will be an investigation of the target of the cyclic GMP in the cells where the concentration changes. A detailed study of the cyclic GMP-binding proteins and cyclic GMP-dependent protein kinase activities in trout testis during spermatogenesis may reveal exciting results.

The induction of a high affinity cyclic AMP phosphodiesterase in trout testis during spermatogenesis (Figure 10B) is not unique to the developing trout testis(93, 94). Identification of, and studies on, the cell type in trout testis in which the induction takes place could provide relevant insights into cyclic nucleotide regulation during spermatogenesis. Investigations of this type should be possible in trout testis, but it may be that studies on the mammalian testis, with better defined endocrinology and cytology may be more fruitful at this time; ideally, detailed studies on both systems should be carried out.

It seems clear from this introductory study that a detailed study of the involvement of cyclic nucleotides in testis development will provide both surprising and interesting results.

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APPENDIX

Various methods for the separation of cyclic nucleotides were investigated during this research (Figure 19). The separation of cyclic AMP from cyclic GMP, in trout testis samples, was routinely performed by elution of AG 1-X8 resin columns with formic acid (Figure 19A). Under the conditions detailed in the legend for Figure 19A, it was found that cyclic GMP contamination in the cyclic AMP fraction was 2%, and cyclic AMP contamination in the cyclic GMP fraction was 4%.

Separation of cyclic AMP and cyclic GMP on DEAE-cellulose columns was also investigated (Figure 19B). While the separation was excellent, this method was too time-consuming for routine sample purifications. On DEAE-cellulose fractionation, with a linear gradient of $0 - 0.1$ M NH_4HCO_3, cyclic AMP and cyclic GMP separated from 5'AMP (Figure 19B). Cyclic AMP, cyclic GMP and 5'AMP eluted at 0.02, 0.04, and 0.06 M NH_4HCO_3, pH 7.8, respectively. Both cyclic AMP and cyclic GMP could be eluted by batch elution with 0.05 M NH_4HCO_3, without elution of 5'AMP.

Separation of cyclic nucleotides, by high pressure liquid chromatography on Partisil-10 SAX, was well investigated, (Figure 20) to determine if this method was sensitive enough to quantitate trout testis cyclic AMP and cyclic GMP. Although cyclic AMP and cyclic GMP separated well on Partisil-10 SAX, the maximum sensitivity obtained under the operating

FIGURE 19

A.   Separation of cyclic AMP and cyclic GMP on a BioRad
AG 1-X8 (formate) 200-400 mesh, resin column (0.35 x 0.7
cm) by elution with formic acid. Elution was performed
under handpump pressure. Column fraction size was 2 ml.
[^{3}H]cyclic nucleotides were chromatographed on identical
separate columns and the results graphed as a composite of
the two elution profiles. Cyclic AMP was eluted with 2 N
formic acid (12 ml; 85% recovery) and cyclic GMP was
eluted with 5 N formic acid (14 ml; 75% recovery). The
prewash was with 0.1 N formic acid (10 ml).

☐   cyclic AMP

▨   cyclic GMP

B.   Separation of cyclic AMP, cyclic GMP and AMP on a DEAE-
cellulose (DE 32) column (0.5 x 10.2 cm) by elution with
a linear gradient from 0 - 0.1 M NH_4HCO_3, pH 7.8. Column
flow rate was 14 ml per hr, total gradient volume was 100
ml and column fraction volume was 2.7 ml. Five absorbance
units of each compound were applied and their specific
elution positions determined by wavelength scans of the
peak tubes. Conductivity was measured in mMHO and con-
verted to NH_4HCO_3 by comparison with standards. Cyclic
AMP recovery was 90 - 100%; cyclic GMP recovery was 85 -
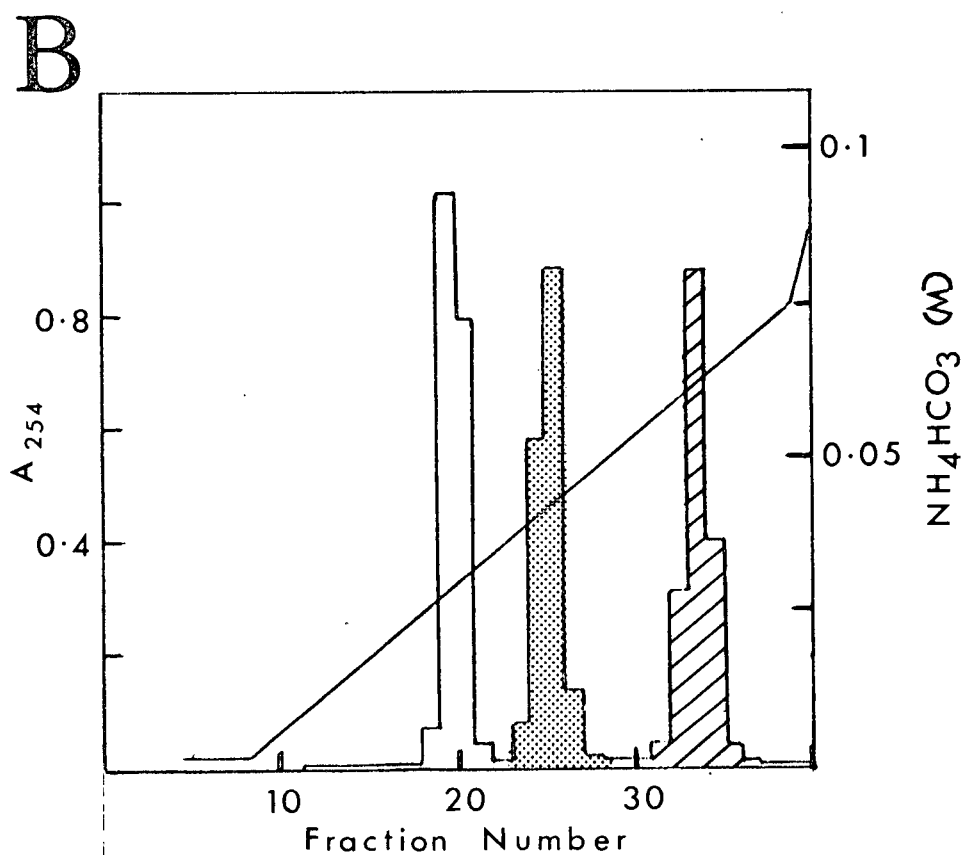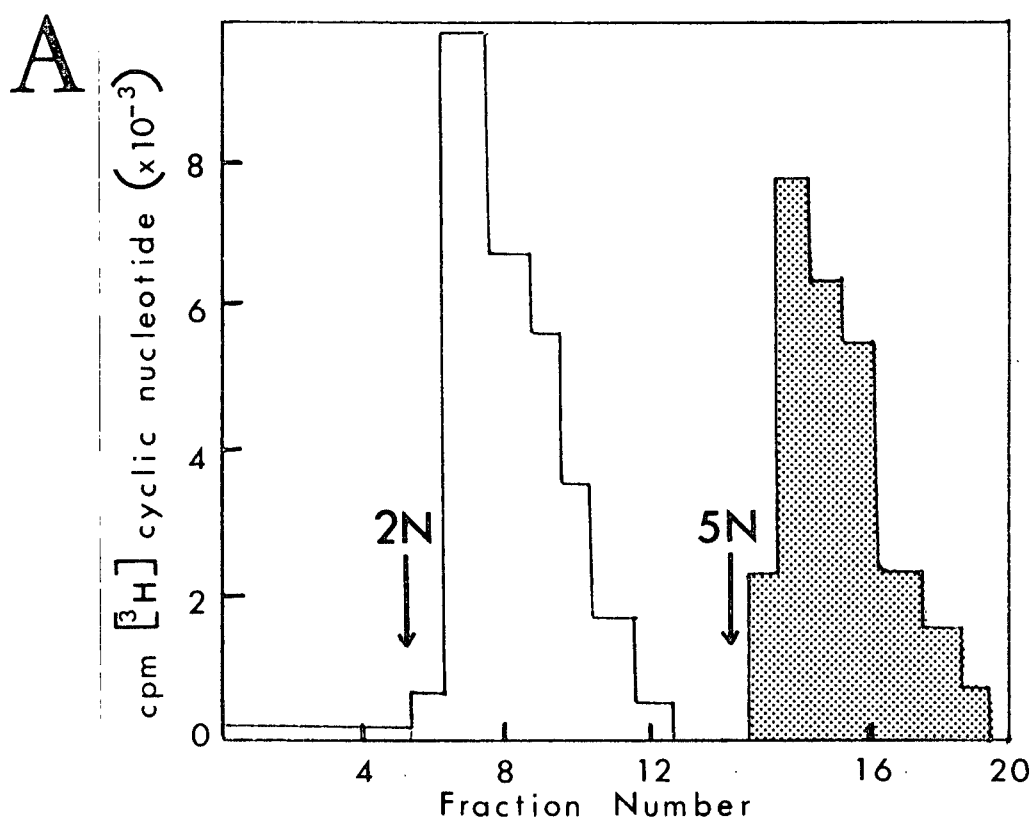90%. Symbols as above, plus:

▨   AMP

FIGURE 20

A. Separation of cyclic nucleotides (A,G,C,U) on Partisil-10

SAX (Reeve-Angel Co., U.S.A). Operating conditions:-

Column: PXS - 1025 SAX; 4.6 mm x 25 cm
Column Temperature: Ambient
Mobile Phase: 0.007 M KH_2PO_4, pH 3.2
Flow Rate: 1.1 ml per min
Pressure: Pump setting 40; 200 - 840 psi
Detection: UV A_{254}nm; 100% sensitivity 0.01 A_{254}nm
Peaks: a. cyclic CMP (0.05 A_{254}nm total)
      b. cyclic AMP   "
      c. cyclic UMP   "
      d. cyclic GMP   "

B. Separation of cyclic nucleotides (A,G,C,U,I) on

Partisil-10 SAX. Operating conditions:-

Column: PXS - 1025 SAX; 4.6 x 25 cm
Column Temperature: Ambient
Mobile Phase: 0.003 M KH_2PO_4, pH 3.2
Flow Rate: 1.1 ml per min
Pressure: 200 - 840 psi
Detection: UV A_{254}nm; 100% sensitivity 0.01 A_{254}nm
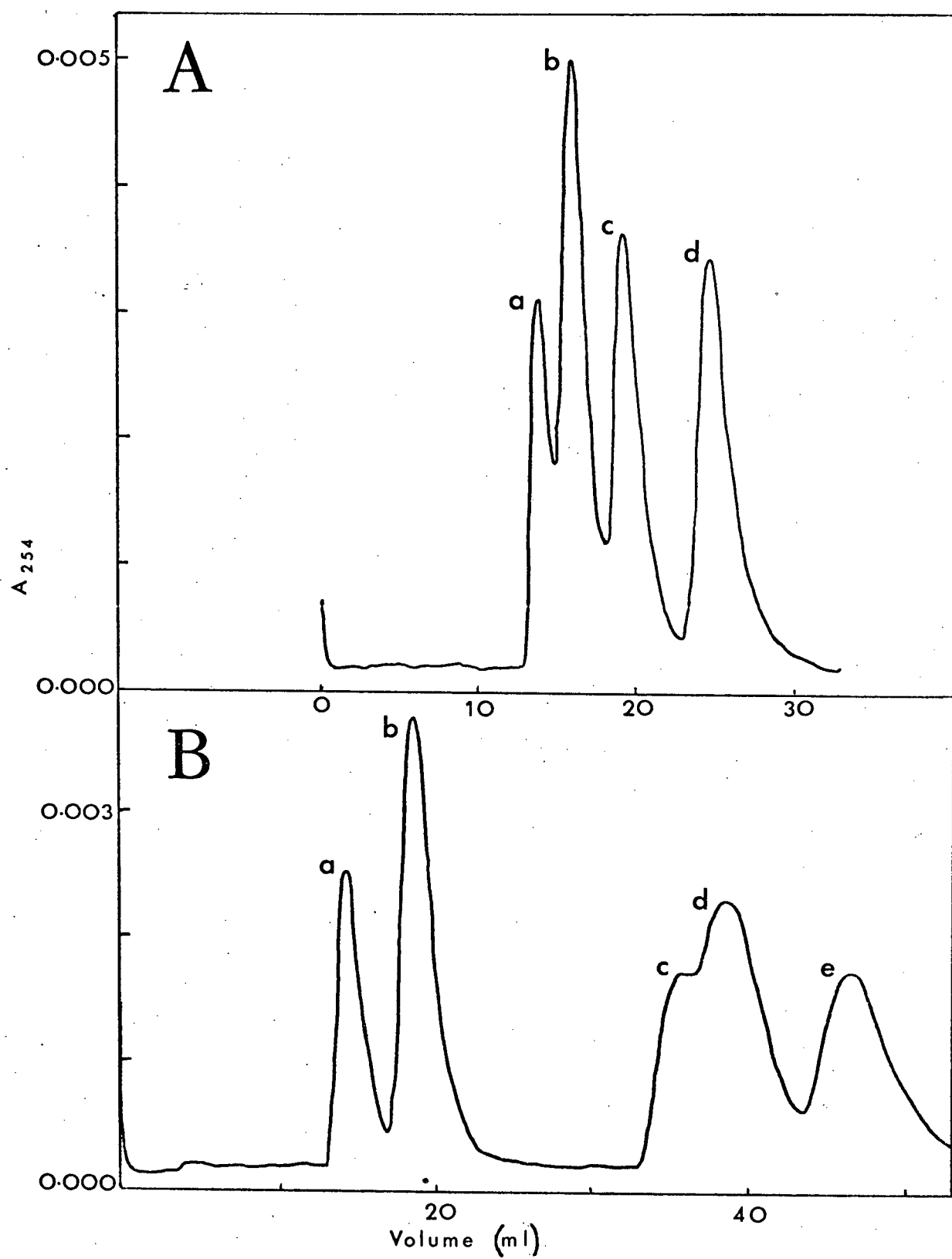Peaks: a. cyclic CMP (0.05 A_{254}nm total)
      b. cyclic AMP   "
      c. cyclic IMP   "
      d. cyclic UMP   "
      e. cyclic GMP   "

conditions used, was 50 pmol/10 μl sample, about 10-100

fold higher than the amount of cyclic AMP or cyclic GMP

in such a sample from trout testis. Furthermore, cyclic

AMP was shown to overlap with 5'CMP and cyclic GMP to

overlap with 5'AMP, as shown in Figures 21A and 21B).

Therefore, complete separation of these nucleotides from

cyclic AMP and cyclic GMP would be required before these

cyclic nucleotides could be quantitated in biological

samples, even if the system's sensitivity was acceptable.

FIGURE 21

A. Separation of 5'CMP, 5'AMP and 5'UMP on Partisil-10

SAX. Operating conditions:-

Column: PXS - 1025 SAX; 4.6 mm x 25 cm

Column Temperature: Ambient

Mobile Phase: 0.005 M KH_2PO_4, pH 3.2

Flow Rate: 1.1 ml per min

Pressure: 200 - 840 psi

Detection: UV A_{254}nm; 100% sensitivity 0.005 A_{254}nm

Peaks: a. 5'CMP (0.13 A_{254}nm total)

      b. 5'AMP (0.05   "   )

      c. 5'UMP (0.13   "   )

B. Separation of cyclic AMP and cyclic GMP on Partisil-10

SAX. Operating conditions as described above.

Peaks: a. cyclic AMP (0.025 A_{254}nm total)

      b. cyclic GMP   "