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ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM

IN ISLETS OF LANGERHANS


A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

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BY

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Oklahoma City, Oklahoma

1972

ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM

IN ISLETS OF LANGERHANS

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## ACKNOWLEDGMENTS

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Cyclic AMP | Adenosine 3', 5'-monophosphate |
| Cyclic CMP | Cytidine 3', 5'-monophosphate |
| Cyclic GMP | Guaninosine 3', 5'-monophosphate |
| Cyclic IMP | Inosine 3', 5'-monophosphate |
| Cyclic TMP | Thymidine 3', 5'-monophosphate |
| Cyclic TuMP | Tubercidin 3', 5'-monophosphate |
| Cyclic UMP | Uridine 3', 5'-monophosphate |
| 5'-AMP | Adenosine 5'-monophosphate |
| ATP | Adenosine **triphosphate** |
| EGTA | Ethyleneglycol-bis($\beta$-aminoethyl ether)-N, N'-tetraacetic acid |
| I. U. | International Unit |
| PEP | Phosphoenolpyruvate |
| PRPP | 5-phosphoribosyl-1-pyrophosphate |
| **ACTH** | **Adrenocorticotropic** hormone |
| TSH | Thyroid-stimulating hormone |
| PG | Prostaglandin |
| mg | Milligram |
| g | Gram |
| **nmole** | Nanomole |
| Tris | Tris (hydroxymethyl) amino methane HCl |
| Km | Michaelis constant |

# LIST OF ABBREVIATIONS- -Continued.

| | |
|---|---|
| v/v | Volume to volume |
| cm | Centimeter |
| min | Minute |
| mM | Millimolar |
| μM | Micromolar |

# ADENOSINE 3', 5'-MONOPHOSPHATE SYSTEM

## IN ISLETS OF LANGERHANS

## CHAPTER I

## INTRODUCTION

Adenosine 3', 5'-monophosphate (cyclic AMP), which is seen below (Fig. 1), was identified chemically by Sutherland et al. (1-3).
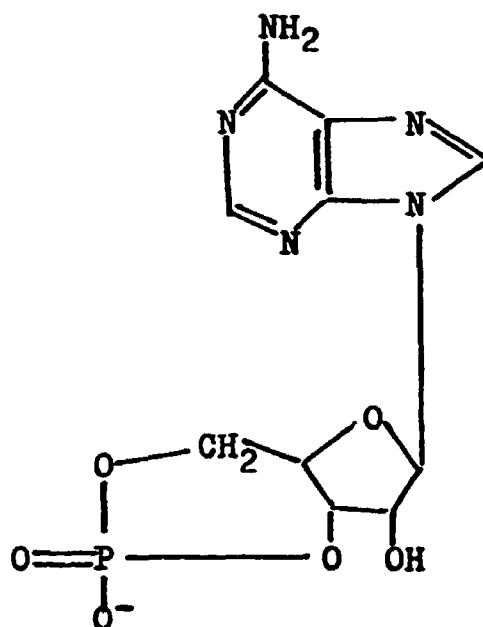


Fig. 1 - Adenosine 3', 5'-monophosphate.

The cellular levels of cyclic AMP are controlled by adenylate cyclase, a membrane bound enzyme (4) which catalyzes its synthesis from adenosine triphosphate (ATP),

and phophodiesterase which mediates its degradation (5).

Since the discovery that cyclic AMP mediates its hyperglycemic effects by the stimulation of glycogen phosphorylase, (1, 6, 7), a great many regulatory functions of cyclic AMP in various tissue have been proved. A model of the protein component of membrane adenylate cyclase, which consists of at least two subunits, a regulatory subunit (R) and a catalytic subunit (C), has been postulated by Robison et al. (8) and is as follows (Fig. 2) :

Extracellular          Cell          Intracellular
                     membrane
                                                    ATP

Hormone ──────────▶   R   │   C   ────▶

                                              Cyclic AMP
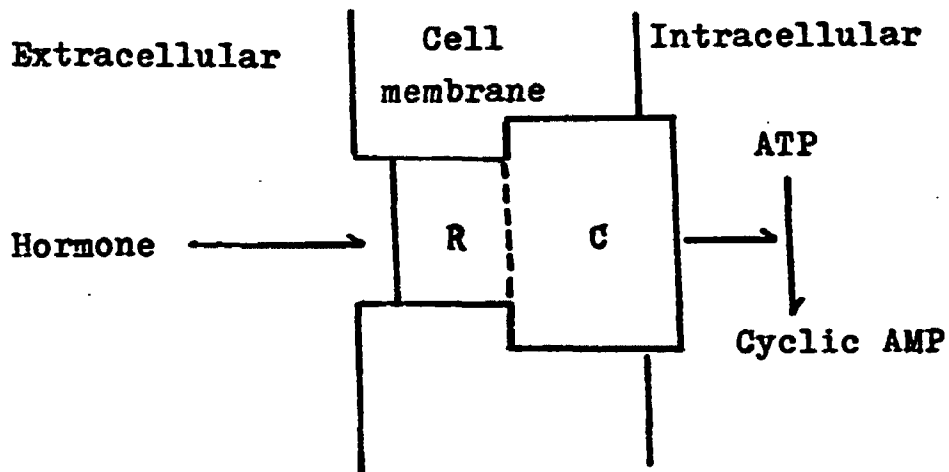
Fig. 2 - Adenylate cyclase system.

In more detail, the regulator subunit is possibly composed of several different parts. The adrenergic ⍺-receptor is in part connected to the catalytic subunit such that interaction with a catecholamine leads to a decrease in adenylate cyclase activity. In contrast, the adrenergic β-receptor mediates an increase in the enzyme activity. However, receptors for other hormones could be located on the same or different subunits (9).

Many hormones, which themselves are the "first messengers" (illustrated on the following page), have been demonstrated to act on their target cells by utilizing cyclic AMP as a "second messenger", the intracellular signal, which may undergo amplification in different ways leading to the ultimate physiological responses (10).

Kuo and Greengard have proposed that all of the varied effects of cyclic AMP results from the phosphorylation of proteins with ATP by cyclic nucleotide dependent protein kinases (11, 12). This hypothesis is supported by recent reports that some protein kinases have a greater affinity for cyclic AMP than cyclic GMP (13), and that histones (14), hormone-sensitive lipase from fat cells (15), microtubular protein from brain (16), glycogen phosphorylase kinase from muscle (17, 18), as well as glycogen synthetase from muscle (19) are phosphorylated by cyclic AMP dependent protein kinase.

Panceatic insulin, a very important polypeptide hormone, serves to decrease hepatic intracellular cyclic AMP levels, while increasing such things as carbohydrate metabolism, glycogen storage, fatty acid synthesis, amino acid uptake, and protein synthesis. Insulin is known to be synthesized by and secreted from the $\beta$-cells of multicellular pancreatic islets. Normally, islets only comprise 1-2% of whole pancreatic mass. A method to isolate these is-
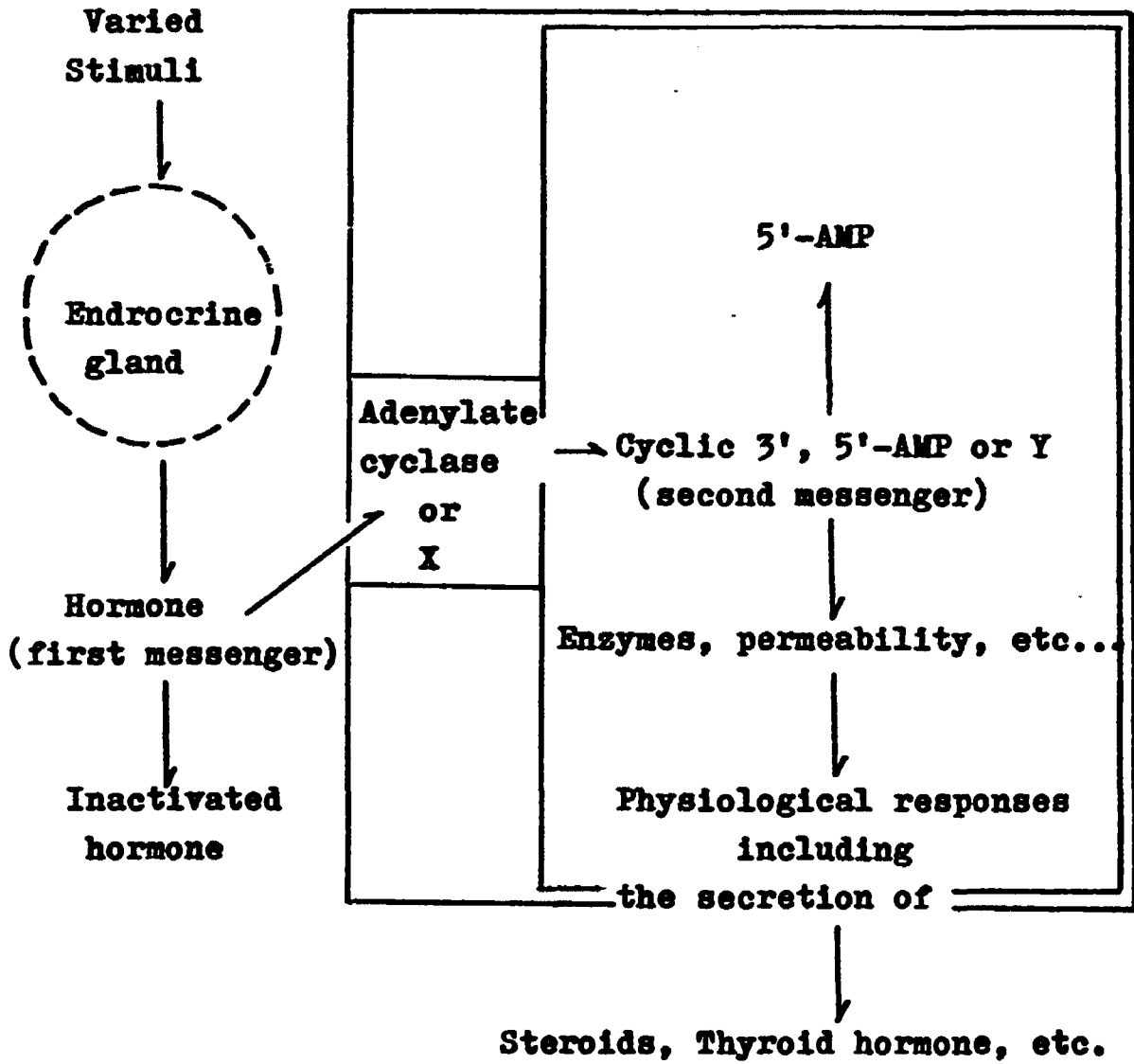
Fig. 3 - Second messenger concept.

lets in sufficient quantities for the study of their phy-
siological and biochemical properties has been elucidated
(20). It is based on disruption of the acinar parenchyma
by injecting buffer solution into the common bile duct. The
gelatinous mass is cut into small pieces and incubated with
crude collagenase. After complete digestion, islets are re-
leased, and islet-rich fractions can be separated from in-
cubation mixtures by sedimentation. The free, round (or
ovoid), greyish-white and intact islets can be recognized
easily under a dissecting microscope when viewed against a
black background. Insulin is secreted in vitro from these
isolated islets which appear normal by electron microscopy
even after 1.5 hour incubation periods.

Various techniques used for the immunoassay of in-
sulin, and the study of the regulation of insulin secretion
both in vivo and in vitro have been disscussed by Mayhew
et al.(21). Metabolizable sugars, certain amino acids, en-
teric hormones, and sufonylureas may initiate insulin sec-
retion. The catecholamines, glucagon, adrenal corticoids,
adrenocorticotropin, growth hormone, thyroid hormone, TSH,
oxytocin, and vasopressin are known to be endocrine reg-
ulators of insulin secretion. However, "neurogenic" control
of insulin secretion is another type of regulation.

Although an important role for cyclic AMP in in-
sulin secretion has been suggested by recent studies, its
actual meachanism remains obscure. The administration of

cyclic AMP enhances insulin secretion from perfused pan-
creas (22). Turtle et al. (23) have shown that in isolated
islets, the changes in the levels of cyclic AMP correlate
well with the pattern of insulin secretion in response to
theophylline, epinephrine, adrenergic blocking agents, and
glucagon. Many other hormones have been found to increase
intracellular levels of cyclic AMP in other tissues. Of
further interest, is the finding that many of the enzymes
involved in glycolysis, the hexose monophosphate shunt, the
Krebs cycle, and in glycogen synthesis are all in islet
tissue (24-29). Cyclic AMP has been found to be involved
in regulation of glycogen synthesis, glucose oxidation,
gluconeogenesis and permeability in certain tissues (30).
Several studies indicate that oxidative metabolism of glu-
cose and energy production is important for insulin secre-
tion (30, 32). From these observations, it can be conclu-
ded that the secretory mechanism of insulin is very com-
plicated and that under certain conditions, cyclic AMP may
be involved.

In this study, the cyclic AMP system in islets of
Langerhans includes adenylate cyclase, phosphodiesterase,
cyclic AMP dependent kinase, and protein kinase modulator.

CHAPTER II

MATERIALS AND METHODS

## Materials

### A. Hormones

The sample of prostaglandins was the kind gift of Dr. J. E. Pike, Upjohn. Leuteinizing hormone was obtained from Schwarz/Mann. Isoproterenol (L and DL form), DL-epinephrine, L-norepinephrine, insulin (24 I.U./mg), ACTH (100 I.U./mg), TSH (1 I.U./mg), pancreozymin, and secretin were purchased from Sigma.

### B. Drugs

The following drugs were obtained from commercial sources and include: KÖ 592 (1-3-methylphenoxyl-2-hydroxy 3-isopropylpropranolamine), C. H. Boehringer and Sohn; phentolamine, Ciba Pharmaceuticals; MJ 1999 (sotalol), Mead Johnson; pilocarpine hydrochloride (4% isoptocarpine), Alcon Laboratory; D-INPEA (N-isopropyl-p-nitrophenyl-ethanolamine), Selvi e C., Italy; DL-propranolol, Sigma.

### C. Enzymes and Amino Acids

The following materials were purchased from Sigma:

L-arginine, L-leucine, and pyruvate kinase. Collagenase (crude) was obtained from Worthington. Cyclic AMP dependent protein kinase from bovine heart was prepared by the procedure of Kuo et al. (12, 33).

## D. Radioactive Chemicals

The following radioactive chemicals were purchased from commercial sources and include: $[8-{}^{14}C]$ ATP (0.33 Ci/mole) and $[2-{}^{3}H]$ adenine (25 Ci/mole), Schwarz/Mann; adenosine-$H^3$(G) 3', 5'-cyclic phosphate (5 Ci/mole), New England Nuclear. $[7-{}^{32}P]$ ATP was prepared by the procedure of Post and Sen (34).

## E. Others

AG-50WX8 (100-200 mesh, hydrogen form) was purchased from BioRad. Phosphoenolpyruvate and all non-radioactive nucleotides were obtained from Sigma. Other chemicals used were all reagent grade.

## Buffers

Three kinds of buffer were employed in digestion or incubation. Hanks' solution (35), without glucose, contained per liter at pH 7.4:

NaCl...................................8000.0 mg

KCl....................................400.0 mg

$Na_2HPO_4 \cdot 2H_2O$.........................60.0 mg

$KH_2PO_4$.................................60.0 mg

$MgSO_4 \cdot 7H_2O$..........................100.0 mg

$CaCl_2$..............................140.0 mg

$MgCl_2.6H_2O$.........................100.0 mg

$NaHCO_3$..............................350.0 mg

Krebs-Ringers bicarbonate buffer, saturated with 95% $O_2$- 5% $CO_2$, v/v, contained per liter:

NaCl...............................7250.0 mg

KCl.................................373.0 mg

$NaHCO_3$............................2180.0 mg

$KH_2PO_4$..............................169.0 mg

$MgSO_4.7H_2O$.........................320.0 mg

$CaCl_2$................................89.0 mg

glucose............................2000.0 mg

The buffer used most often for collagenase digestion was Rodbell's Tris-salts solution (35) at pH 7.4 and contained per liter:

NaCl...............................7192.0 mg

KCl.................................298.0 mg

$KH_2PO_4$.............................272.0 mg

$CaCl_2·2H_2O$.........................147.0 mg

$MgCl_2.6H_2O$.........................305.0 mg

Tris-HCl...........................3026.0 mg

## Isolation of Pancreatic Islets
### (For Adenylate Cyclase Assay)

Three to seven male Sprague-Dawley rats (about 200 g each) were used for each experiment. Each rat was intra-peritoneally injected with 0.3 ml of 4% pilocarpine just

three hours before surgery. The surgical procedure and
cannulation of the common bile duct was similar to that
of Lacy (20), except that 10 ml of Rodbell's Tris-salts
buffer, pH 7.4 (36), was injected into the pancreas instead
of Hanks' solution (35). The pancreas was then removed,
trimmed, chopped into small pieces, washed several times
with the Tris-salts buffer and allowed to settle. Ten mg
of collagenase for each pancreas was added to the sediment
after removal of the supernatent fluid and the mixture was
stirred at $37^o$ for 20 min.

Following this digestion, the mixture was poured
onto a 180 mesh silk cloth and washed with 200 ml of cold
Tris-HCL (40 mM) -MgSO$_4$ (5 mM), pH 7.4. The cloth was then
inverted and the islet-rich tissue washed into a tube with
200-300 ml of the same solution. After several min the is-
lets had settled to the bottom of the tube and the super-
natant fluid was discarded. The islets were then trans-
ferred to a cold Petri dish and viewed with microscopic
aid (10 x). Large islets were siphoned through an 18 gauge
metal needle into a test tube or glass homogenizer. The
islets were homogenized with four or five strokes of a
tight fitting ground glass homogenizer. A Lowry protein
assay (37) was performed for each experiment and the pro-
tein content estimated by comparison with bovine serum
albumin. The protein content was consistently 1.7 $\pm$ 0.1 $\mu$g
per islet.

## Adenylate Cyclase Assay

Basically, the assay system involves the following reactions (Fig. 4):

PEP        Pyruvate
           Kinase

Radioactive ADP ────────────→ Radioactive ATP
   +Pi
                  ←───────
              ATPase        $Mg^{++}$   Adenylate
                                        Cyclase of
                                        Islet Homogenate

                                        ↓
      Phosphodiesterase       Radioactive
5'-AMP ────────X──────────    Cyclic AMP
               ¦
      Theophylline
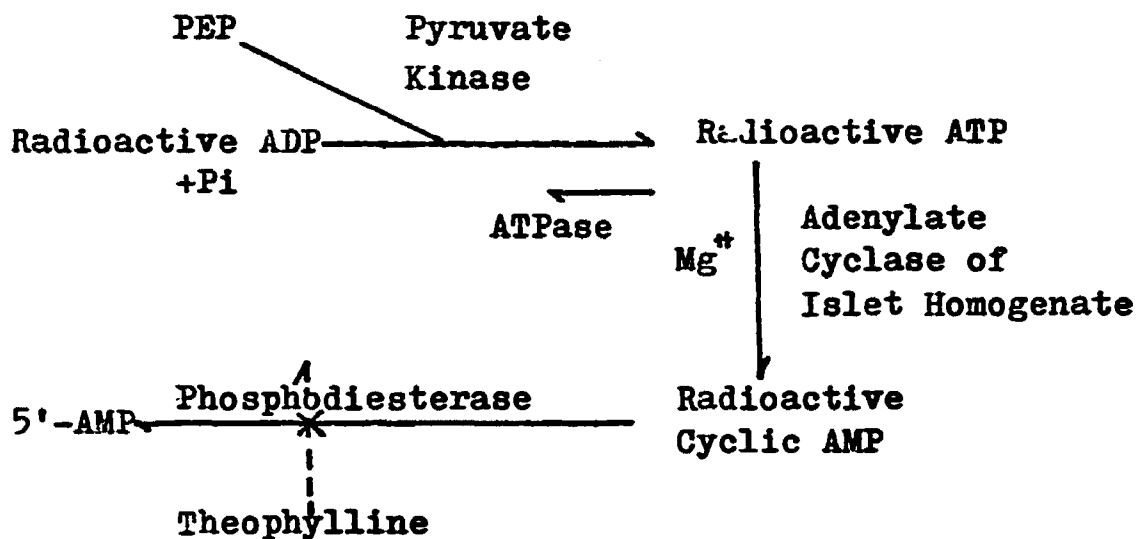
Fig. 4 - Reactions involved in adenylate cyclase assay.

The standard incubation mixture, in a final volume of 0.95 ml, consisted of the following: 40 mM Tris-HCl containing 5 mM $MgSO_4$ (adjusted to pH 7.4 with 5 N NaOH); 0.5 mM cyclic AMP; 1 mM /8-$^{14}$C/ ATP (0.33 Ci/mole); 10 mM theophylline; 5 mM phosphoenolpyruvate; 9 I.U. of pyruvate kinase; 85 to 100 μg islet protein; and test substances where indicated. This mixture was incubated at $30^o$ for 10 min. Unless otherwise stipulated, all experiments, including the controls in which no islet homogenate was added, were performed by incubating the islet samples in Triplicate for each type of treatment. Each incubated sample was then separately assayed for the amount of radioactive cyclic AMP synthesized.

The reactions were terminated by the addition of
0.1 ml 50% trichloracetic acid, followed by centrifugation
and neutralization of the supernatant solution with 0.1 ml
of 2.5 M Tris base. Next, 0.05 ml $ZnSO_4$ (5%) and 0.05 ml
$Ba(OH)_2$ (2.56%) were added to the supernatant solution and
the mixture was centrifuged (5,000 x g, 10 min). The su-
pernatant fluid was then lyophilized overnight. The dried
samples were dissolved in 0.5 ml water and applied to co-
lumns (0.5 x 2.5 cm) of AG-50WX8 (Bio-Rad, hydrogen form,
100-200 mesh). The columns were then eluted with water,
and the third and fourth ml of eluate used as the cyclic
AMP fraction. The optical density at 260 mμ of this frac-
tion was monitored during the first 40 individual enzyme
assays (Figs. 7 and 8) and found to consisently reflect a
cyclic AMP recovery of 75-80%. The recovery of cyclic AMP
was also estimated to be 72-78% when 1 nmole of adenosine-
$H^3(G)$ 3', 5'-cyclic phosphate (containing about 2 x $10^6$cpm)
was added to the incubated islet samples immediately follow-
ing the addition of trichloracetic acid for the purpose of
determining the recovery of the cyclic nucleotide. In all
succeeding experiments the value of 75% recovery was used.
The entire cyclic AMP fractions (2 ml each) were then mixed
with 18 ml of Aquasol (New England Nuclear) and the radio-
activity was counted in a liquid scintillation spectrometer.
In all cases, with 85 to 100 μg islet protein (representing
50-60 islets) the control experiments produced about 2

nmoles of cyclic AMP per min per mg protein after subtrac-
tion of the negligible zero time control (0.1-0.2 nmole).
This procedure for assaying adenylate cyclase activity is
a modification of that developed by Krishna et al. (38),
and was found to result in minimal contamination of the
cyclic AMP fractions with the radioactive ATP. The authen-
ticity of cyclic AMP in the AG-50WX8 eluate was verified
by the following experiment. The cyclic AMP sample obtained
by the above procedure was chromatographed with standard
cyclic AMP on a thin layer plate (SilicAR TLC-7GF, Mallin-
ckrodt), using a solvent system consisting of isopropyl
alcohol-$H_2O$-29% $NH_4OH$ (7:3:1, v/v). The radioactivity re-
covered from the cyclic AMP spot on the developed plate
represented the total radioactivity originally present in
the cyclic AMP sample.

The results of all experiments are presented as the
means and standard deviation for the triplicate samples of
each set of treatments.

### Prelabeling of Pancreatic Islets

Intact islets were used in this procedure. The
principle of prelabeling is based on (Fig. 5):

Radioactive Adenine  
+  
Intact Islets  
+  
PRPP (of Islets)

}———→ Intracellular Radioactive 5'-AMP

↓

Intracellular Radioactive ATP

| Stimulants or Inhibitors of Cyclic AMP System of Islets

Radioactive Cyclic AMP
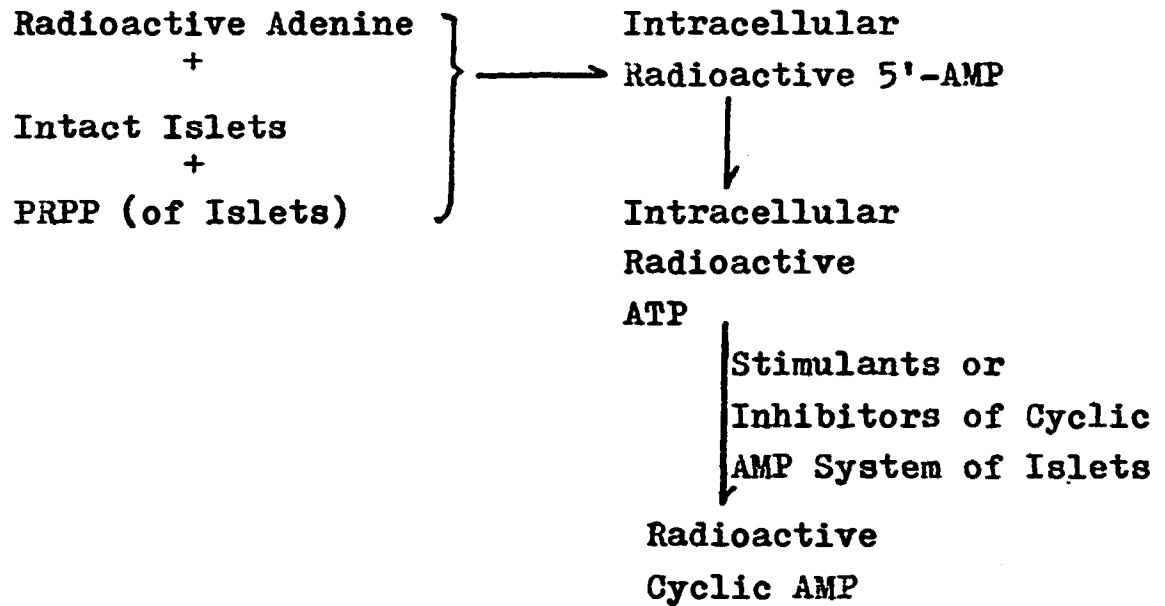
Fig. 5 - Reactions involved in prelabeling method.

Two methods were employed for prelabeling the islets. The first method (Figs. 10-12) consists of simultaneously digesting pancreatic pieces with crude collagenase and prelabeling the islets. Pancreatic pieces obtained from 2 male Sprague-Dawley rats (about 200 g each) were incubated, with stirring, at $37^o$ in 2 ml of Rodbell's Tris-salts solution (36) in the presence of 20 mg of crude collagenase and 36 µ Ci of $[2-^3H]$ adenine for 30 minutes. The digested tissue was then treated, and intact islets isolated, according to the procedures described previously. The collected islets were washed once with 30 ml of cold Kreb-Ringer bicarbonate buffer (previously saturated with 95% $O_2$-5% $CO_2$,v/v, and containing no glucose), and were finally suspended in an appropriate volume of the same buffer to yield a suspension containing 30-50 islets per ml.

The second method (Tables 8-11) consists of digesting the pancreatic tissue followed by prelabeling the isolated islets from five rats. The procedures used to digest pancreatic pieces and isolate islets were exactly the same as described before. About 2,000 islets were suspended in 4 ml of Krebs-Ringer bicarbonate buffer (previously saturated with 95% $O_2$- 5% $CO_2$, v/v) containing 50 μCi of [2-$^3$H] adenine. The islets were incubated for 20 min at 37°, with shaking, at the end of the prelabeling period, 30 ml of cold Krebs-Ringer buffer (containing no glucose) was added and the mixture was centrifuged at 2,000 r.p.m. for 3 minutes. The supernatant solution was then discarded. The washing procedure was repeated 5 times, and the islets were finally transferred to an appropriate volume of same buffer to yield a suspension containing 20-40 islets per ml.

In experiments (Table 12) in which the absolute levels of total cyclic AMP were measured by the protein kinase catalytic method (33), the islets were isolated as in the second method, with the omission of the prelabeling step. The islets were suspended in an appropriate volume of Krebs-Ringer bicarbonate buffer to yield a suspension containing 45 islets per ml.

## Incubation of Islets and Measurement of Cyclic AMP Levels

In experiments (Figs. 10-12; Tables 8-11) in which the relative levels of radioactive cyclic AMP in islet

cells were measured, appropriate volumes of the islet sus-
pension were incubated at $37^o$ for different periods in the
presence of various agents, as indicated in the legends to
the figures and the tables. At the end of the incubation
period, one-tenth volume of 50% trichloracetic acid was
added to the incubation mixture followed by the addition
of 0.02 ml of 1 μM cyclic AMP as a carrier. The islet sus-
pension was then sonicated for 0.5 min, then centrifuged
at 2,000 r.p.m. for 5 min. The precipitate was removed and
the supernatant fluid was then neutralized with 1 M Tris.
The radioactive cyclic AMP in the supernatant fluid was
purified by the same procedure described in the adenylate
cyclase assay. One ml of the pooled cyclic AMP fraction
was assayed for radioactivity.

The absolute amounts of cyclic AMP (Table 12) were
measured by cyclic AMP dependent protein kinase catalytic
method (33). The experimental procedure was based on the
following reaction (Fig. 6):

Histone
+
$[\gamma\,^{32}P]$ ATP
$\left.\begin{array}{l}\\ \\ \\ \end{array}\right\}$
$\underrightarrow{Mg^+, \text{ Cyclic AMP}}$ Histone—$^{32}P$+ADP
Cyclic AMP
Dependent
Protein Kinase
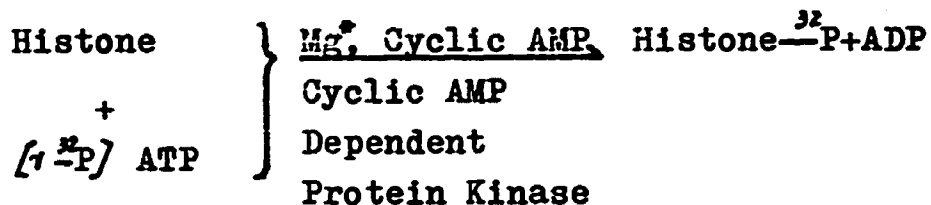
Fig. 6 - Reaction involved in cyclic AMP dependent
protein kinase catalytic method .

The incubation mixture used for the assay contained,
in a final volume of 0.2 ml; sodium acetate buffer, pH 6.0,

10 $\mu$moles; magnesium acetate, 2 $\mu$moles; histone mixture, 40 $\mu$g; $[\gamma - {}^{32}P]$ ATP, 1 nmole, containing about 2.1 x $10^6$ cpm; heart protein kinase, 3 $\mu$g. Incubations were carried out at 30$^o$ for 5 min in a shaking water bath. The reaction was terminated by addition of 2 ml of 5% trichloracetic acid containing 0.25% sodium tungstate, pH 2.0 (39); 0.1 ml of 0.63% bovine serum albumin was added as a carrier protein. After standing at 0$^o$ for 5 min, the mixture was centrifuged, and the supernatant solution removed by aspiration. The precipitate was dissolved in 0.1 ml of 0.5 N NaOH, and 2 ml of 5% trichloracetic acid-0.25% sodium tungstate solution, pH 2.0, was added. This mixture was centrifuged followed by aspiration of the supernatant described before. The above procedure was repeated once more. The protein was finally collected by centrifugation and dissolved in 0.1 ml of 0.5 N NaOH, and the radioactivity was measured in a liquid scintillation counter. The amount of cyclic AMP present in the samples was determined from standard curves with known quantities of cyclic AMP.

CHAPTER III

RESULTS

## Isolation of Pancreatic Islets

The effects of pilocarpine usage on the yield of
large islets as compared to other procedures, are presented
in Table 1. In author's hands, treatment of rats with pilo-
carpine facilitated more than a 10-fold increase in the
yield of pancreatic islets, i.e. from 25-35 islets per pan-
creas from the untreated rats to 350-500 islets from the
treated rats. When the amount of collagenase used was in-
creased 6-fold and the volume of the digestion mixture was
increased 4- to 8-fold by the addition of Hanks' solution,
the yield of islets was less than one-half of our usual
value. The procedure described here,to the author's knowledge,
yields the greatest amount of islets per pancreas, and more-
over, the islets isolated have been shown to possess adeny-
late cyclase responsive to various agents which are known to
stimulate insulin secretion.

Another method to isolate islets by discontinous
dextran density gradients (2.5%, 12%, and 22%; 2.5%, 8%,
14%, 16%, 18.5%, and 22%. at 1000xg centrifugation for 10

TABLE 1

THE EFFECT OF PILOCARPINE PRETREATMENT ON THE YIELD OF ISLET ISOLATED
FROM RAT PANCREAS

| Pretreatment | Buffer used with collagenase digestion | Digestion time | Collagenase concentration | Yield of islets |
|---|---|---|---|---|
| | | min | mg/ml | number of islets/pancreas |
| None | 4 ml Hanks' solution | 15 | 60 | 80-150 |
| None | 4 ml Rodbell's Tris-salts solution | 15 | 60 | 80-150 |
| None | None | 20 | 10 | 25-35 |
| Pilocarpine | None | 20 | 10 | 350-500 |

Method for isolation of pancreatic islets was as described under
"Experimental Procedure" except for some modifications as indicated in
the table.

minute) was also tried. Although islets could be isolated
between the 12% and 22% density layers (or between the
16% and 18.5% density layers), the total yield of islets
was quite poor. Unfortunately, islets could not be frozen
and stored to accumulate large quantities for study. Only
most of phosphodiesterase activity was found to be stable,
while adenylate cyclase activity was rapidly lost (data
not shown).

## Adenylate Cyclase Activity In
## Isolated Pancreatic Islets

A. Dependence of Cyclic AMP Production on Incubation
Time and Islet Protein Concentration

Fig. 7 illustrates the time course of the reaction
at five and ten minutes in the presence and absence of the
polypeptide enteric hormone, secretin. The control reponse
is roughly linear with time while the stimulated enzyme
further increases its activity in the second 5 min. period,
i.e., a 49% stimulation at 5 min. and 130% at 10 min.

The dependence of cyclic AMP production upon islet
protein concentration is demonstrated in Fig. 8. A linear
relationship was noted both in the presence and absence of
secretin. Similar linearity was also observed with experi-
ments in which the adenylate cyclase preparation was in-
cubated with glucagon and ACTH (data not shown).

Fig. 7. The dependency of cyclic AMP production
upon incubation time. The assays were performed under
standard conditions with 0.102 mg protein/ml, in the
absence (•) or presence (o) of 20 μg/ml secretin.

22
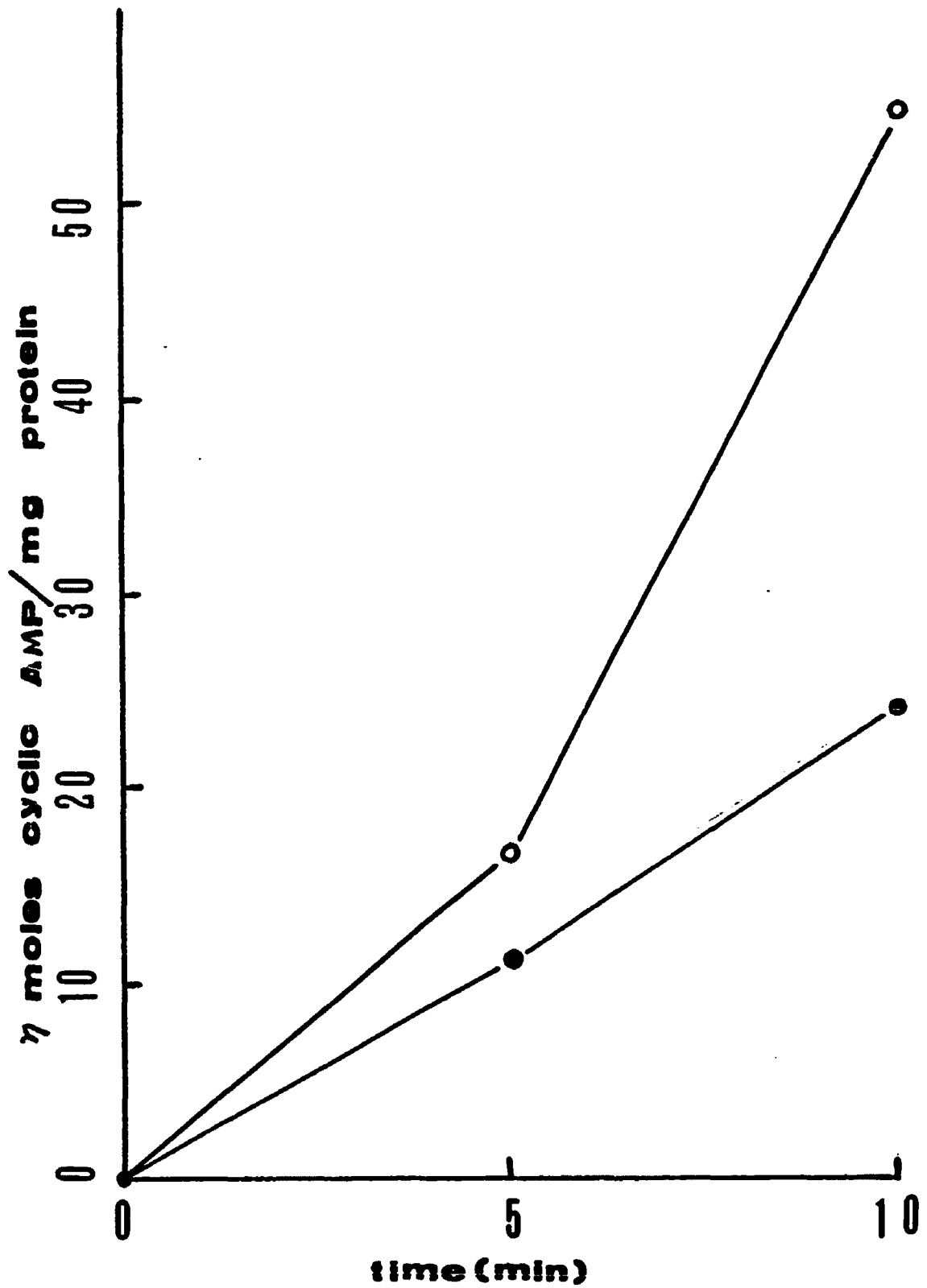


Fig. 7 – The dependency of cyclic AMP production upon incubation time.

Fig.8 . The dose-dependency of cyclic AMP pro-

duction as a function of the amount of adenylate cyclase.

The assays were performed under the standard conditions

except for the variation of enzyme amount, in the absence

(•) and presence (o) of secretin (20 μg/ml). Each point

shown represents mean of the values obtained with tri-

plicate samples.
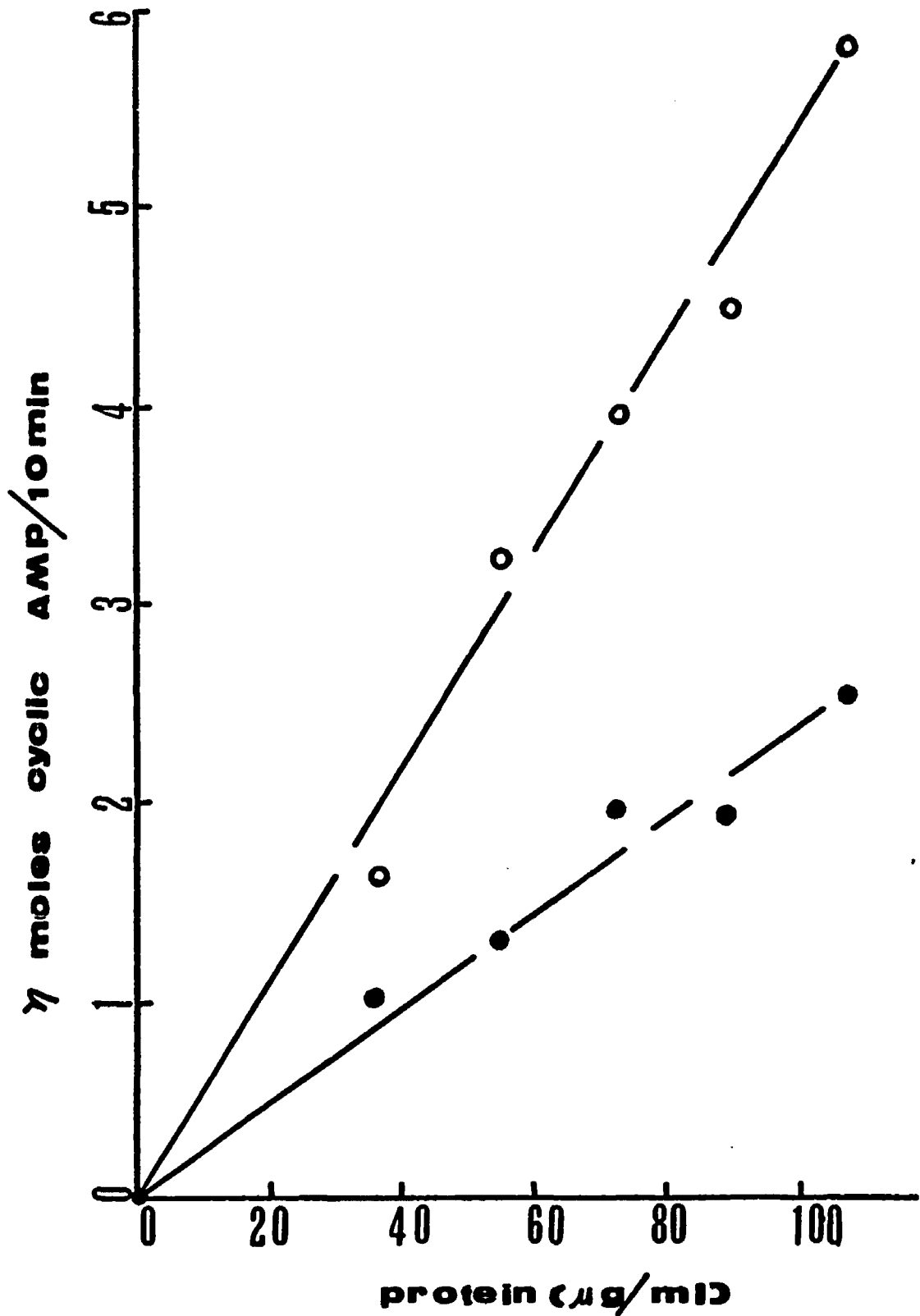
Fig. 8 - The dose-dependency of cyclic AMP pro-
duction as a function of the amount of adenylate cyclase.

B. The Effect of Polypeptide Hormones

The main purpose of the present study was to ascertain the relationship between the activation of islet adenylate cyclase and the stimulation of insulin secretion. Glucagon, previously found to stimulate insulin secretion in isolated islets (40) and other pancreatic tissue preparations (41, 42), stimulated islet adenylate cyclase. Maximal stimulation of islet adenylate cyclase was found to occur at about 30 $\mu$M glucagon (Fig. 9) compared with 2 $\mu$M for adenylate cyclase of adipocytes (43) and 0.1 $\mu$M for that of liver cells (44). Turtle and Kipnis (23), using intact rat islets, have reported that glucagon, in the presence of theophylline, causes an increase in islet cyclic AMP content. Their results are consistent with our observations, although the intact cells are apparently more sensitive to hormone than is the adenylate cyclase of islet homogenates.

In view of the recently published observations by Rodbell et al. (45) that guanosine phosphates augment the effect of glucagon in stimulating liver cell adenylate cyclase. We studied the effects of GTP and GDP on the islet enzyme. While glucagon alone (1 $\mu$M) had virtually no effect on enzyme activity and 5 $\mu$M GDP alone gave a 51% stimulation, the combination of the two agents resulted in a 118% stimulation (Table 2). A ten fold increase in GDP concentration (50 $\mu$M) resulted in a 146% stimulation of adeny-

Fig. 9 . The effect of glucagon upon islet adenylate cyclase activity. The assays were performed under standard condition at the indicated glucagon concentrations. The basal level of activity (with no added glucagon), which amounts to 2.3 nmoles cyclic AMP produced per min per mg islet protein, was normalized to 100. Each point shown represents a value obtained from a single incubation.
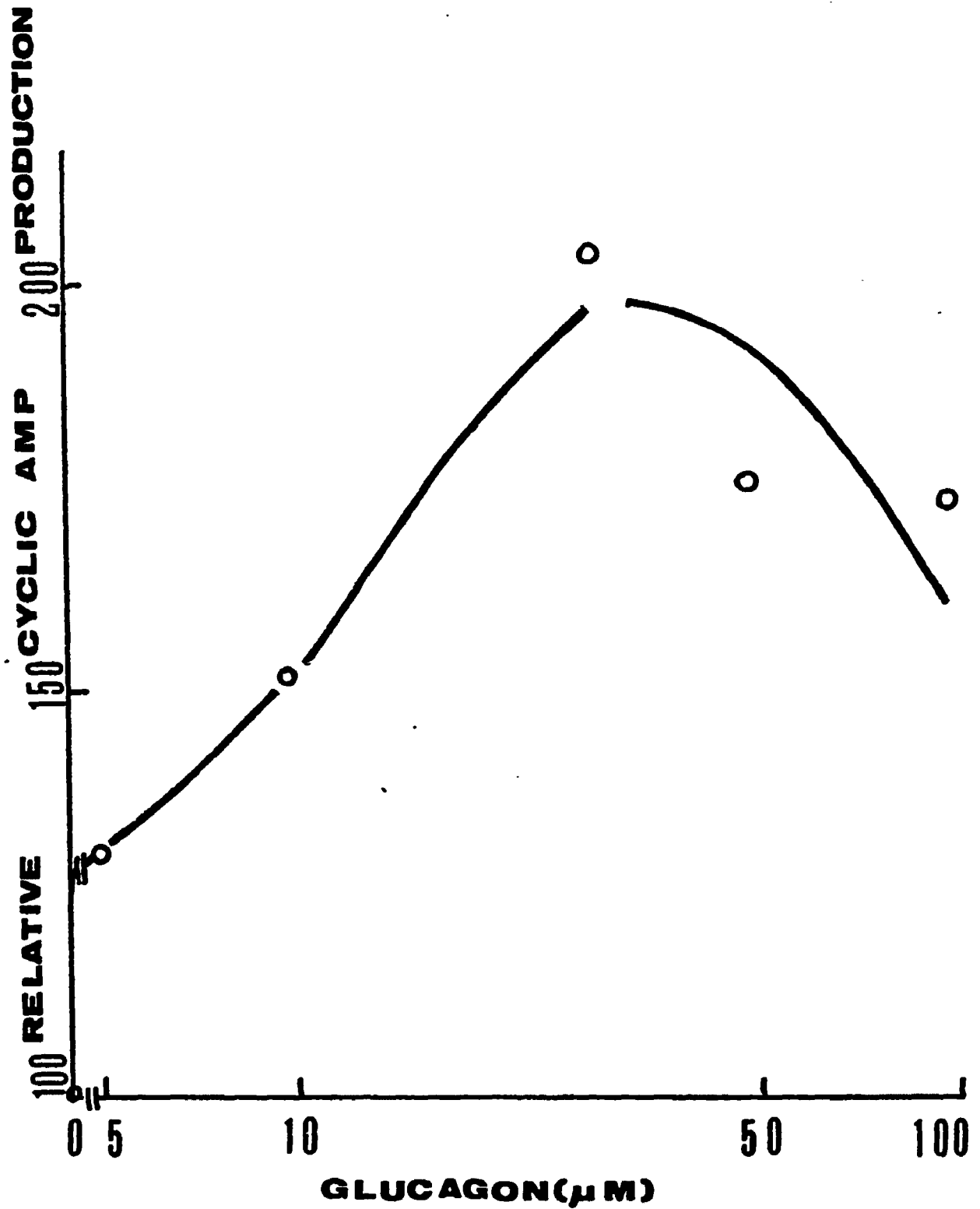
Fig.9 -The effect of glucagon upon islet adenylate cyclase activity.

Table 2. Effects of glucagon, GDP, GTP, and EGTA, present singly or in combination on the islet adenylate cyclase activity. The incubation conditions were as described under "Experimental Procedure". The control (without added agent) value, represents $2.01 \pm 0.05$ nmoles cyclic AMP per min per mg islet protein. Each value shown represents mean $\pm$ standard deviation of triplicate samples.

## TABLE 2

EFFECTS OF GLUCAGON, GDP, GTP, AND EGTA, PRE-
SENT SINGLY OR IN COMBINATION ON THE
ISLET ADENYLATE CYCLASE ACTIVITY

| Addition and concentration | Relative cyclic AMP production |
|---|---|
| None (control)................................. | 100.0 ± 2.5 |
| Glucagon (5.0 μM)............................ | 129.2 ± 4.3 |
| EGTA (1.0 mM)................................. | 121.0 ± 4.6 |
| Glucagon (5.0 μM) + EGTA (1.0 mM) ............................ | 251.9 ± 8.2 |
| Glucagon (1 μM)............................... | 100.0 ± 2.1 |
| GDP (5 μM)................................... | 151.2 ± 4.2 |
| Glucagon (1 μM) + GDP (5 μM) ............................ | 218.5 ± 8.9 |
| GDP (50 μM).................................. | 246.8 ± 6.7 |
| GTP (5 μM)................................... | 218.9 ±16.6 |
| Glucagon (1 μM) + GTP (5 μM) ............................ | 181.5 ±20.1 |

late cyclase activity in the absence of glucagon. GTP, at
a concentration of 5 $\mu$M, stimulated the enzyme activity
119%, whereas in the presence of 1 $\mu$M glucagon, the sti-
mulation was reduced to 81%. Rodbell et al. (45, 46) found
that in liver the glucagon-stimulated adenylate cyclase
activity is further augmented by both GDP and GTP under
their experimental conditions.

At a concentration of 1 mM, the calcium chelating
agent EGTA stimulated the basal activity of islet adenylate
cyclase by 21%. It was found to synergistically enhance
the stimulatory effects of 5 $\mu$M glucagon from 20% in its
absence to 152% in its presence (Table 2). Similar results
have been obtained by Hepp et al. (47) with liver adeny-
late cyclase. They postulated that the EGTA stimulation is
due to the chelation of inhibitory membrane-bound calcium
ions.

Other polypeptide hormones were also tested for
their effects on islet adenylate cyclase (Table 3). The
two gut hormones, secretin (also see Figs. 7 and 8) and
pancreozymin, were both found to be effective stimulants.
These hormones have been reported to stimulate insulin
release from pieces of rabbit pancreas (48, 49), but are
ineffective in stimulating insulin release from isolated
rat islets (50) and other pancreatic tissue preparations
(51, 52). Neither of the hormone preparation used in the

experiments was pure; the secretin (Sigma, Type $\text{I\!I}$) contain-
ing 25-30 units of pancreozymin per 100 units of secretin,
and the pancreozymin (Sigma, Grade $\text{I\!I}$), containing about
10% secretin. The results obtained with the two hormone
preparations could be due to the action of either one of
them, or of both. Further elucidation of this point re-
quires the use of pure hormone preparations.

ACTH activated islet adenylate cyclase to a greater
extent than did any other hormone (Table 3). This hormone
has been found to enhance insulin secretion from isolated
rat pancreas (22, 42) and increase plasma insulin levels
in mice (53). TSH, previously shown to augment the glucose
-induced insulin secretion from pieces of rat pancreas
(42), was also found to activate islet adenylate cyclase.
Somatotropin also increased the activity of islet adeny-
late cyclase. This hormone has been found to elevate canine
serum levels of insulin in vivo (54), but has not been
shown to increase insulin secretion in vitro (31, 55).
Luteinizing hormone was not active at the concentration
tested. Insulin at 20 µg per ml significantly inhibited
islet adenylate cyclase, suggesting that insulin may reg-
ulate the islet cyclic AMP levels (and thus insulin secre-
tion from the organelles) through a feed-back control me-
chanism.

Table 3 . Effects of polypeptide hormones on islet
adenylate cyclase activity. Experimental conditions were
as under "Experimental Procedure". The control (without
added agent) value, represents $2.35 \pm 0.11$ nmoles cyclic
AMP produced per min per mg islet protein. Each value shown
represents mean $\pm$ standard deviation of triplicate sample,
except that the value obtained with luteinizing hormone
was from a simple sample.

## TABLE 3

## EFFECTS OF POLYPEPTIDE HORMONES ON ISLET
## ADENYLATE CYCLASE ACTIVITY

| Additions and concentrations | Relative cyclic AMP production |
|---|---|
| None (control) | $100.0 \pm 4.7$ |
| ACTH (46 I.U./ml) | $361.3 \pm 23.2$ |
| ACTH (23 I.U./ml) | $508.9 \pm 16.3$ |
| Somatotropin (100 $\mu$M) | $181.8 \pm 6.6$ |
| Somatotropin (50 $\mu$M) | $224.3 \pm 14.3$ |
| TSH (20 $\mu$g/ml) | $204.2 \pm 14.9$ |
| Pancreozymin (20 $\mu$g/ml) | $186.7 \pm 4.7$ |
| Luteinizing Hormone (20 $\mu$g/ml) | $101.2$ |
| Insulin (20 $\mu$g/ml) | $74.8 \pm 6.1$ |
| Insulin (10 $\mu$g/ml) | $87.7 \pm 8.2$ |

C. Effects of Amino Acids, Alcohol,

Acetylcholine and NaF

Several amino acids have been found to stimulate insulin release from pieces of rabbit pancreas. Milner (56) has shown, for example, that arginine enhances glucose-induced insulin secretion and that leucine, even in the absence of glucose, causes insulin release. Theophylline was found to enhance leucine-induced but not arginine-induced secretion. In the present study, neither of these amino acids were found to stimulate islet adenylate cyclase (Table 4). In fact, they slightly inhibited enzyme activity, suggesting that cyclic AMP may not mediate their insulinogenic effect.

Ethanol was found to stimulate islet adenylate cyclase (Table 4). The lowest level tested (0.05% or 8.6 mM) was found to give a 59% stimulation, whereas the stimulation was reduced at higher ethanol concentrations. Various alcohols have been found to stimulate adenylate cyclase of both rat liver and hamster insulinoma (insulin producing islet adenoma) (57). Ethanol, for example, was found to stimulate the hepatic enzyme but was not tested with the insulinoma. 2-Propanol, which stimulated insulinoma adenylate cyclase, also enhanced the glucagon response of this enzyme. Clinical observations have indicated that ethanol administration (oral or intravenous) results in an increase in the plasma insulin response to

Table 4 . Effects of leucine, ethanol and acetyl-
choline on islet adenylate cyclase activity. The incubation
conditions were as described under "Experimental Procedure".
The control (without added agent) value, represents $1.88 \pm$
$0.07$ nmoles cyclic AMP produced per min per mg islet pro-
tein. Each value shown represents mean $\pm$ standard deviation
of triplicate samples.

## TABLE 4

## EFFECTS OF LEUCINE, ETHANOL AND ACETYLCHOLINE ON ISLET ADENY- LATE CYCLASE ACTIVITY

| Addition and concentration | Relative cyclic AMP production |
|---|---|
| None (control) | $100.0 \pm 3.7$ |
| L-leucine (5.0 mM) | $73.1 \pm 3.3$ |
| L-leucine (2.5 mM) | $80.5 \pm 3.6$ |
| L-arginine (5.0 mM) | $50.8 \pm 3.2$ |
| L-arginine (2.5 mM) | $64.7 \pm 1.9$ |
| Ethanol, 0.5% (v/v) | $105.9 \pm 2.9$ |
| Ethanol, 0.1% (v/v) | $128.7 \pm 5.1$ |
| Ethanol, 0.05% (v/v) | $159.5 \pm 2.6$ |
| Acetylcholine (0.1 mM) | $279.1 \pm 12.3$ |
| NaF (10.0 mM) | $405.5 \pm 28.7$ |

glucose. Ethanol alone, however, did not cause an elevation of plasma insulin levels (58, 59). Thus, ethanol stimulation of islet adenylate cyclase may enhance glucose-initiated insulin secretion.

Cholinergic drugs have been found to stimulate the release of insulin from slices of rat pancreas (60, 61). Acetylcholine has been shown to stimulate the release of insulin from isolated rat islets in the presence of sub-optimal glucose levels (1.5 mg/ml) (40). The finding (Table 4) that acetylcholine stimulates islet adenylate cyclase is consistent with these observations. To the author's knowledge, adenylate cyclase stimulation by acetylcholine has not been found with other tissues. Sodium fluoride greatly stimulated islet adenylate cyclase (Table 4), as noted with many other tissue preparations.

### D. Effects of Glucose and Hypoglycemic Drugs

Glucose was found to have a slight inhibitory effect upon islet adenylate cyclase at 0.5-5.0 mM (Table 5), concentrations known to initiate insulin secretion (44). The data from the present study are similar to those reported by Atkins and Matty with mouse islets (62). These results are consistent with an intracellular mechanism of secretory stimulation by this metabolite, especially since glucose freely permeates islet cells (63, 64).

Tolbutamide, 1-butyl-3-(p-tolylsulfonyl) urea, a

Table 5. Effects of glucose and hypoglycemic drugs on islet adenylate cyclase activity. Experimental conditions were as described under "Experimental Procedure". The control (without added agent) value, represents $2.26 \pm 0.06$ nmoles cyclic AMP produced per min per mg islet protein. Each value shown represents mean $\pm$ standard deviation of triplicate samples.

## TABLE 5

### EFFECTS OF GLUCOSE AND HYPOGLYCEMIC DRUGS
### ON ISLET ADENYLATE CYCLASE ACTIVITY

| Addition and concentration (mM) | Relative cyclic AMP production |
|---|---|
| None (control) | $100.0 \pm 2.7$ |
| D-glucose (5.0 mM) | $94.3 \pm 2.4$ |
| D-glucose (1.0 mM) | $97.8 \pm 2.7$ |
| D-glucose (0.5 mM) | $100.1 \pm 2.5$ |
| Tolbutamide (0.5 mM) | $273.7 \pm 20.1$ |
| Tolbutamide (0.1 mM) | $234.6 \pm 26.2$ |
| Phenformin (0.1 mM) | $105.6 \pm 2.3$ |

common clinically utilized drug for the treatment of dia-
betes, was found to effectively stimulate islet adenylate
cyclase; 0.1 and 0.5 mM concentrations of the hypoglycemic
drug gave 135 and 174% stimulation, respectively (Table 5 ).
This drug has been found to stimulate insulin secretion
both in vitro with isolated rat islets (65) and in vivo
(66, 67). Levey et al. have recently reported that tolbu-
tamide stimulates adenylate cyclase of rabbit heart (68)
and islet cell adenoma (69). Tolbutamide has also been
shown to inhibit phosphodiesterase activity in an insulin-
secreting islet cell tumor of Syrian golden hamster (70)
and increase cyclic AMP level in fat cells (71). However,
Hellman et al. (72) have presented evidence that tolbu-
tamide does not enter the $\beta$-cells and therefore must have
an extracellular site of action.

Another hypoglycemic drug, phenformin (1-phen-
ethylbiguanidine), thought to act intracellularly and
differently from tolbutamide (73), had no effect on islet
adenylate cyclase (Table 5).

E. Effects of Prostaglandins and Adrenergic Agents

Prostaglandins $E_1$, $E_2$, $A_1$, and $F_{1\alpha}$, each tested
at a concentration of 20 µg/ml, significantly enhanced
the production of cyclic AMP (Table 6). In vivo insulin
secretion is induced by the prostaglandins (74), although
prostaglandin $E_1$ (1 µg/ml), in the presence of 1.5 mg/ml

glucose, fails to stimulate the secretion of insulin by isolated rat islets (40).

Atkins and Matty (62) have presented evidence that epinephrine and isoproterenol, in the presence of phento-lamine (an $\alpha$-adrenergic blocker), increase adenylate cy-clase activity of microdissected mouse islet tissue. They reported that the stimulation by epinephrine in the pre-sence of $\alpha$-blocker is inhibited by propranolol (a $\beta$-adren-ergic blocker). They suggested that stimulation of islet adenylate cyclase is a $\beta$-adrenergic function while inhi-bition of the cyclase activity is an $\alpha$-adrenergic function.

Montague and Cook (44) have reported that epine-phrine inhibits glucose-induced insulin secretion and lowered the intracellular cyclic AMP content of isolated islets. Epinephrine and norepinephrine were also shown to inhibit insulin secretion in vitro with a hamster pancreas preparation (75). Turtle and Kipnis (23) have observed that epinephrine plus phentolamine, but not epinephrine plus propranolol, increses of cyclic AMP levels in intact islets.

In the present studies with rat islet adenylate cyclase, epinephrine and norepinephrine were both found to inhibit its activity by about 14-17%; this inhibition was increased to about 35-37% in the presence of KÖ 592, a $\beta$-adrenergic blocking agent (Table 6). Conversely, both catecholamines, in the presence of phentolamine, activated

Table 6. Effects of epinephrine, norepinephrine, isoproterenol, KÖ 592, phentolamine, and prostaglandins, present singly or in combination on islet adenylate cyclase activity. Experimental conditions were as described under "Experimental Procedure". The control (without added agent) value, represents $2.14 \pm 0.04$ nmoles cyclic AMP synthesized per min per mg islet protein. Each value shown represents the mean $\pm$ standard deviation of triplicate samples.

TABLE 6

EFFECTS OF EPINEPHRINE, NOREPINEPHRINE, ISOPROTERENOL, KÖ 592, PHENTOLAMINE, AND PROSTAGLANDINS ON ISLET ADENYLATE CYCLASE ACTIVITY

| Addition and concentration | Relative cyclic AMP production |
|---|---|
| None (control)...........................100.0 $\pm$ 1.9 | |
| DL-epinephrine (0.1 mM)...................83.0 $\pm$ 3.6 | |
| DL-epinephrine (0.1 mM) + KÖ 592 (0.1 mM) ...............63.2 $\pm$ 8.0 | |
| DL-epinephrine (0.1 mM) + phentolamine (0.1 mM) .............446.6 $\pm$19.8 | |
| L-norepinephrine (0.1 mM)..................86.1 $\pm$ 4.8 | |
| L-norepinephrine (0.1 mM) + KÖ 592 (0.1 mM) ...............65.1 $\pm$ 6.7 | |
| L-norepinephrine (0.1 mM) + phentolamine (0.1 mM).................394.5 $\pm$11.5 | |
| L-isoproterenol (0.1 mM)...................452.1 $\pm$27.7 | |
| L-isoproterenol (0.1 mM) + KÖ 592 (0.1 mM) ................151.6 $\pm$25.0 | |
| PG $E_1$ (20 $\mu$g/ml)............................212.2 $\pm$32.7 | |
| PG $E_2$ (20 $\mu$g/ml)............................167.1 $\pm$18.5 | |
| PG $A_1$ (20 $\mu$g/ml)............................150.3 $\pm$16.8 | |
| PG $F_{1\alpha}$ (20 $\mu$g/ml)..........................160.7 $\pm$28.4 | |

adenylate cyclase by about 300%. Isoproterenol alone great-
ly activated the enzyme and this activation was largely
abolished by KÖ 592, indicating that the activation by
isoproterenol was characteristically a $\beta$-effect.

F. Effects of 3', 5'-Cyclic Nucleotides

Cyclic AMP, cyclic UMP, and cyclic IMP were found
to have inhibitory effects on islet adenylate cyclase at
concentrations higher than 2 mM. Cyclic GMP, even when
tested at a concentration of 0.1 mM, tremendously inhibited
adenylate cyclase activity. It could be due to the inhibi-
tion of ATP binding. However, both cyclic TMP and cyclic
CMP did not significantly inhibit the production of cyclic
AMP (Table 7).

Regulation of Cyclic AMP Levels in
Intact Pancreatic Islets

Glucagon, isoproterenol, ACTH, and acetylcholine,
agents known to stimulate insulin release, all increased
the accumulation of radioactive cyclic AMP in intact rat
pancreatic islets incubated in the presence of 10 mM theo-
phylline (Fig. 10). The accumulation of cyclic AMP in all
cases was reasonably proportional to the incubation time.
It has been shown that these hormones also stimulated
adenylate cyclase in islet homogenates. The cyclic AMP
level for the control experiment (in which no stimulatory

Table 7. Effects of 3', 5'-cyclic nucleotides on
islet adenylate cyclase activity. Experimental conditions
were as described under "Experimental Procedure". The con-
trol (without added agent) value, represents $1.88 \pm 0.99$
nmoles cyclic AMP synthesized per min per mg islet protein.
Each value shown represents the mean $\pm$ standard deviation
of triplicate samples.

TABLE 7

EFFECTS OF 3', 5'-CYCLIC NUCLEOTIDES ON
ISLET ADENYLATE CYCLASE ACTIVITY

| Addition and concentration | Relative cyclic AMP production |
|---|---|
| None (control)................................. | $100.0 \pm 4.5$ |
| Cyclic AMP (2 mM)............................. | $86.6 \pm 2.7$ |
| Cyclic AMP (3 mM)............................. | $67.7 \pm 3.1$ |
| Cyclic GMP (0.1 mM).......................... | $0.0 \pm 2.5$ |
| Cyclic UMP (2 mM)............................. | $75.8 \pm 3.4$ |
| Cyclic IMP (2 mM)............................. | $71.4 \pm 9.5$ |
| Cyclic IMP (3 mM)............................. | $57.9 \pm 11.0$ |
| Cyclic TMP (2 mM)............................. | $102.8 \pm 14.0$ |
| Cyclic TMP (3 mM)............................. | $92.8 \pm 6.4$ |
| Cyclic CMP (3 mM)............................. | $100.6 \pm 5.8$ |

Fig. 10. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time in the presence of a high concentration of theophylline. About 20 islets were incubated for varying time intervals in 0.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. The radioactive cyclic AMP found in the zero time sample was about 3200 cpm per mg protein. X, control; □ , glucagon (0.1 mM); ■ , DL-isoproterenol (0.1 mM); ○, corticotropin (0.05 mM); ●, acetylcholine (0.1 mM).

Fig. 10 - Stimulation by some normones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time.

agents were added) at the end of the 10-min incubation was higher than the zero-time value. With similar experiments in which the islets were incubated in the presence of 3 mM theophylline, the levels of cyclic AMP found in the control and the hormone-treated islet samples were considerably lower than the zero-time value (Fig. 11), suggesting an extremely high phosphodiesterase activity in rat islets. In order to insure a positive increment in the cyclic AMP level during the incubations. The islets were incubated with 10 mM theophylline in all experiments.

The dose-dependent stimulation by glucagon, acetylcholine, isoproterenol and ACTH of cyclic AMP accumulation in the islets is illustrated in Fig. 12. EGTA, which slightly increased the cyclic AMP level when present alone, acted synergistically with glucagon to greatly increase the cyclic AMP levels, confirming these observations of their synergistic effect on adenylate cyclase activity of islets.

The effects of some other agents on the islet cyclic AMP levels are presented in Table 8. Secretin and prostaglandins ($E_1$, $E_2$, $A_1$ and $F_{1\alpha}$), which have been reported to have an insulin-releasing action (48, 49, 74), were all capable of elevating cellular cyclic AMP levels. Thyroid-stimulating hormone, with an unclear insulinogenic effect, was also active. It is interesting that tolbutamide, an oral hypoglycemic drug, caused an elevation of cyclic AMP in islets. The level of islet cyclic AMP was

50

Fig. 11. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of incubation time in the presence of a low concentration of theophylline. About 20 islets were incubated for varying time intervals in 0.5 ml of Kreb-Ringer bicarbonate buffer containing 3 mM theophylline. The radioactive cyclic AMP found in zero time sample was about 3200 cpm per mg protein. X, control; □, glucagon (0.1 mM); ■, DL-isoproterenol (0.1 mM); o, acetylcoline (0.1 mM).
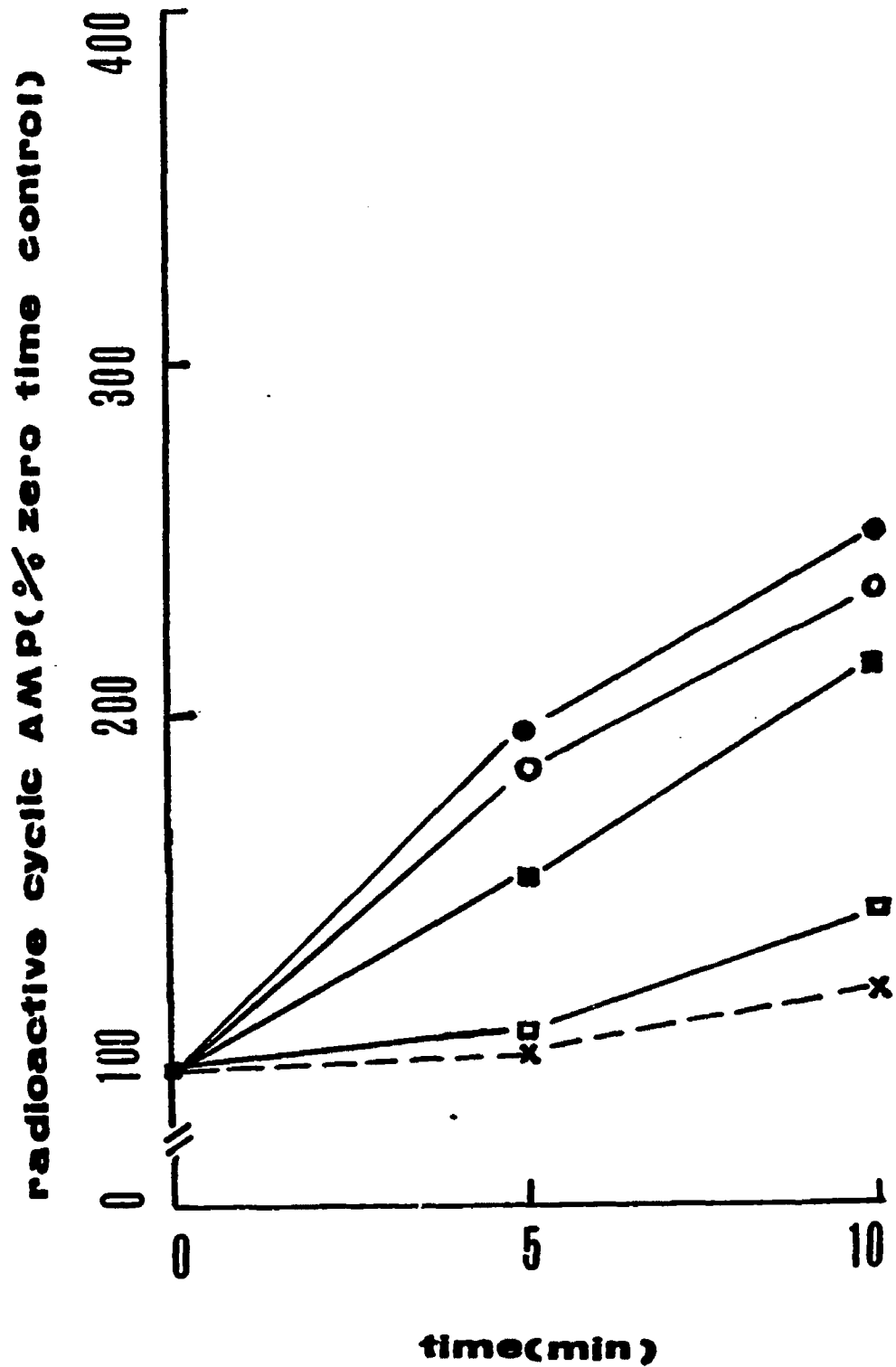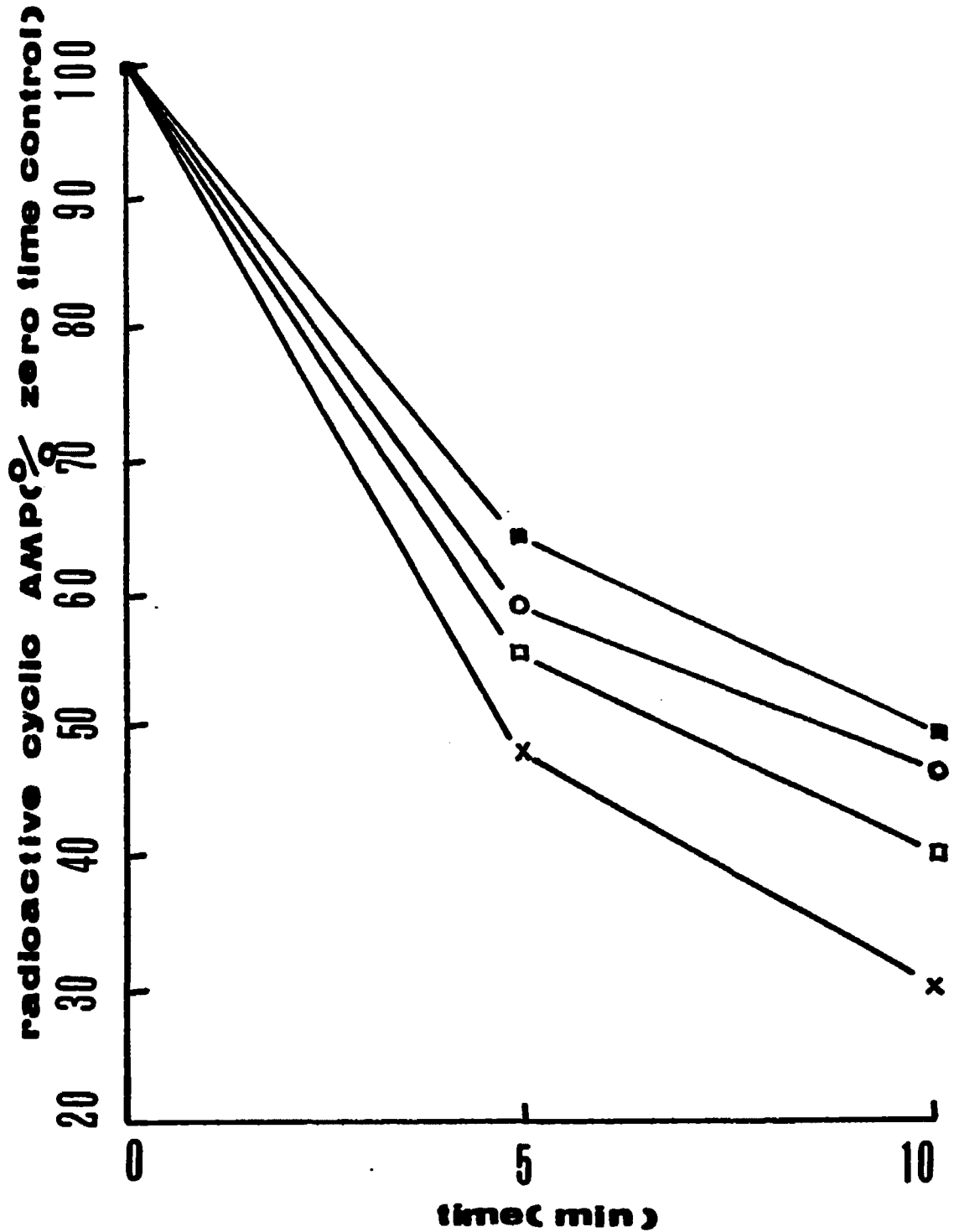
Fig. 11 - Stimulation by some hormones of radio-active cyclic AMP accumulation in rat pancreatic islets as a function of incubation time.

Fig.12. Stimulation by some hormones of radioactive cyclic AMP accumulation in rat pancreatic islets as a function of hormone concentration. About 20 islets were incubated, in the presence of varying the concentration of hormones, for 10 min in 0.5 ml of Krebs–Ringer bicarbonate buffer containing 10 mM theophylline. The basal value (found in the absence of added hormones) of radioactive cyclic AMP at the end of 10 min incubation was about 3000 cpm per mg protein. O, glucagon; ●, glucagon + EGTA (2 mM); ●,DL-iso_proterenol; ▢ , corticotropin; X, acetylcholine.
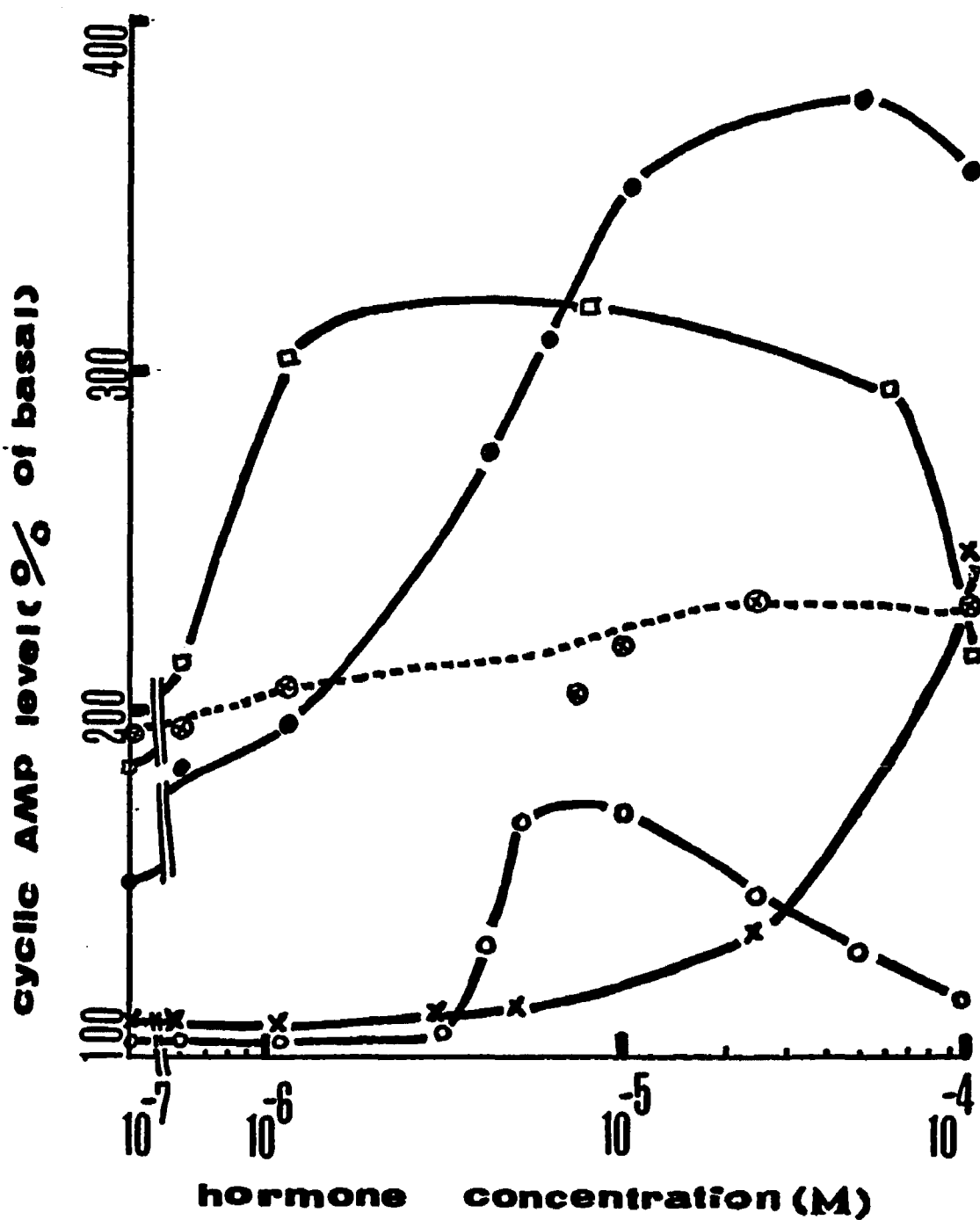
Fig. 12 – Stimulation by some hormones of radio-
active cyclic AMP accumulation in rat pancreatic islets
as a function of hormone concentration.

Table 8 . Effects of prostaglandins, secretin, thyroid-stimulating hormone, tolbutamide, leucine and glucose on cyclic AMP levels in pancreatic islets. About 70 islets were incubated in 2.5 ml of Krebs-Ringer bicarbonate buffer containing 10 mM theophylline and various additives, at $37^{o}$ for 10 min, with shaking. Each incubation was performed at least in duplicate and the mean and the individual value (in parentheses) are presented.

## TABLE 8

EFFECTS OF PROSTAGLANDINS, SECRETIN, TSH, TOLBUTAMIDE, LEU-
CINE AND GLUCOSE ON CYCLIC AMP LEVELS IN PANCREATIC ISLETS

| Additive | Cyclic AMP found |
|---|---|
| | $cpm \times 10^2/mg$ protein |
| None (control)................... | 21.3 (22.0;20.5) |
| Prostaglandin $E_1$ (10 μg/ml).... | 61.9 (57.7;66.0) |
| (20 μg/ml).... | 72.0 (66.2;78.0;81.8;61.8) |
| (30 μg/ml).... | 79.9 (85.0;74.8) |
| Prostaglandin $E_2$ (20 μg/ml).... | 26.5 (23.4;29.6) |
| (30 μg/ml).... | 81.0 (77.1;84.9) |
| Prostaglandin $A_1$ (20 μg/ml).... | 49.3 (38.3;60.3) |
| (30 μg/ml).... | 62.4 (70.1;54.7) |
| Prostaglandin $F_{1\alpha}$ (20 μg/ml)... | 96.3 (86.3;106.2) |
| (30 μg/ml)... | 46.8 (44.3;49.2) |
| Secretin (20 μg/ml)............... | 43.1 (40.7;44.2) |
| TSH (20 μg/ml)................... | 35.9 (32.2;39.6) |
| Tolbutamide (0.1 mM)............. | 37.5 (27.5;49.3;40.2;33.0) |
| Leucine (5 mM)................... | 23.4 (22.6;24.3) |
| Glucose (5 mM)................... | 21.5 (22.1;20.8) |

not significantly increased by leucine or glucose, suggest-
ing that the action of these metabolites to stimulate in-
sulin secretion may not be mediated through a mechanism
involving cyclic AMP. The effects of these agents on islet
cyclic AMP levels confirmed the findings previously made
with adenylate cyclase in the islet homogenates.

It has been shown (23, 62) that rat pancreatic is-
lets contain both $\alpha$-and $\beta$-adrenergic receptors. Interac-
tion of agents with the $\alpha$-receptor causes a reduction in
adenylate cyclase activity and consequently a decrease in
cellular cyclic AMP. Interaction of agents with the $\beta$-re-
ceptor, on the other hand, results in an activation of
adenylate cyclase and an increase in cyclic AMP levels.
The effect of isoproterenol in increasing islet cyclic AMP
level was effectively antagonized by all four of the $\beta$-
adrenergic blockers (propranolol, MJ 1999, KÖ 592, and D-
INPEA) tested (Table 9). It is clear that activation of
adenylate cyclase and the consequent stimulation of insulin
secretion (76) by isoproterenol is a typical $\beta$-adrenergic
effect. Epinephrine and norepinephrine caused reductions
in islet cyclic AMP levels (Table 10), in agreement with
the known effect of these hormones in inhibiting insulin
release (31, 44). In the presence of $\alpha$-adrenergic blocking
agents, such as phenoxybenzamine and phentolamine, the in-
hibition by these two hormones was overcome, and pronounced
increases in cyclic AMP due to these hormones were ulti-

Table 9. Inhibition by $\beta$-adrenergic blocking agents of increase in islet cyclic AMP. About 90 islets were incubated in 2.5 ml of Krebs-Ringer buffer containing 10 mM theophylline and various additives at $37^o$, with shaking, for 10 min. Each incubation was performed in duplicate and the mean and the individual values (in parentheses) are presented.

## TABLE 9

### INHIBITION BY β-ADRENERGIC BLOCKING AGENTS OF INCREASE IN ISLET CYCLIC AMP

| Additive | Cyclic AMP found | Effect of agent |
|---|---|---|
| | cpm x $10^2$/mg protein | % control |
| None (control)............19.5 (20.6; 18.9) | | 100 |
| DL-Isoproterenol (0.2 mM)............417.3 (344.5; 490.0) | | 2141 |
| DL-Isoproterenol (0.2 mM) + DL-propranolol (0.08 mM)............98.1 (100.0; 96.1) | | 503 |
| DL-Isoproterenol (0.2 mM) + MJ 1999 (20 μg/ml)........101.7 (93.6; 109.8) | | 522 |
| DL-Isoproterenol (0.2 mM) + Kö 592 (0.1 mM)............19.8 (21.5; 18.1) | | 102 |
| DL-Isoproterenol (0.2 mM) + D-INPEA (0.4 mM).108.7 (83.9; 133.4) | | 557 |

Table 10. Comparison of effect of epinephrine and norepinephrine on islet cyclic AMP levels in the absence and presence of $\alpha$-adrenergic blocking agents. About 65 islets were incubated in 2.5 ml Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. Each incubation was performed in duplicate and the mean and the individual values (in parentheses) are presented.

## TABLE 10

### COMPARISON OF EFFECT OF EPINEPHRINE AND NOREPINEPHRINE ON ISLET CYCLIC AMP LEVELS IN THE ABSENCE AND PRESENCE OF $\alpha$-ADRENERGIC BLOCKING AGENTS

| Additive | Cyclic AMP found |
|---|---|
| | cpm x $10^2$/mg protein |
| None (control)..................;.....—..........20.1 (19.0;21.2) | |
| DL-Epinephrine (0.05 mM)..................1.9 (3.4;0.4) | |
| DL-Epinephrine (0.05 mM)<br>+ Phenoxybenxamine (0.03 mM)........89.9 (79.8;99.9) | |
| DL-Epinephrine (0.05 mM)<br>+ Phentolamine (0.04 mM).............87.3 (82.2;92.4) | |
| L-Norepinephrine (0.05 mM)..............5.3 (8.0;2.5) | |
| L-Norepinephrine (0.05 mM)<br>+ Phenoxybenzamine (0.03 mM)........ 26.9 (30.7;23.0) | |
| L-Norepinephrine (0.05 mM)<br>+ Phentolamine (0.04 mM)............101.5 (87.5;115.5) | |

mately noted. It seems that the action of epinephrine and norepinephrine in the absence of phenoxybenzamine and phentolamine is predominantly an 𝛼-adrenergic effect, while the action of these hormones becomes a typically 𝛽-adrenergic one in the presence of the 𝛼-adrenergic blockers. The results obtained with the present study employing intact islets confirmed both the findings of Turtle and Kipnis (23) with intact islets, and the studies of islet adenylate cyclase.

It has been shown that GTP enhances glucagon-stimulated adenylate cyclase activity in rat liver plasma membrane preparations (45) and rat islet homogenates, and that GTP itself also stimulates the enzyme activity in these two systems (45). In the present study with intact islet cells, GTP was found to cause increases in islet cyclic AMP levels; the stimulation seen with 10 $\mu$M GTP being much higher than that seen with 5 $\mu$M GTP (Table 11). The stimulatory effect of 5 $\mu$M GTP and 5 $\mu$M glucagon was additive. The stimulation due to 10 $\mu$M GTP, on the other hand, was inhibited by 10 $\mu$M glucagon. The cyclic AMP level was, however, still higher in the presence of both GTP and glucagon than in the presence of glucagon alone, the results shown here with 10 $\mu$M GTP are the same is seen with 5 $\mu$M GTP.

Most of the agents whose effects on islet cyclic AMP levels were studied with the prelabeling method (which measures the relative cyclic AMP levels) were also re-

## TABLE 11

STIMULATORY EFFECT OF GLUCAGON AND GTP, PRESENT SINGLY
OR IN COMBINATION, ON THE ACCUMULATION
OF CYCLIC AMP IN PANCREATIC ISLETS.

| Additive | Cyclic AMP found | Effect of agent |
|---|---|---|
| | cpm/mg protein | % control |
| None (control) | 19.7 (19.2;20.1) | 100 |
| Glucagon (5 µM) | 47.8 (43.2;52.3) | 243 |
| GTP (5 µM) | 25.4 (21.8;28.9) | 129 |
| Glucagon (5 µM) + GTP (5 µM) | 57.7 (57.2;58.2) | 293 |
| Glucagon (10 µM) | 40.9 (43.6;38.2) | 208 |
| GTP (10 µM) | 310.4 (300.1;320.7) | 1576 |
| Glucagon (10 µM) + GTP (10 µM) | 73.7 (67.1;80.2) | 374 |

About 80 islets were incubated in 2.5 ml of Krebs-
Ringer bicarbonate buffer containing 10 mM theophylline
and various additives, at $37^o$ for 10 min, with shaking.
Each incubation was performed in duplicate and the mean
and the individual values (in parentheses) are presented.

examined with the protein kinase catalytic method (which measures the absolute cyclic AMP levels) (Fig. 13). The results, presented in Table 12, confirm all the findings made with the prelabeling method. It is worth mentioning that EGTA, showing a small stimulatory effect itself, synergistically enhanced the stimulation evoked by glucagon. The potentiation of glucagon action by EGTA was also seen with the prelabeling experiments (Fig. 12), and also with adenylate cyclase in islet homogenates. The basal level of cyclic AMP in isolated islets (49.0 pmoles/mg protein, see footnote to Table 12), about 3-to 10-fold higher than the value found in many other tissues (33), was comparable to that reported by Turtle and Kipnis (23). Enhancement of intracellular cyclic AMP levels were as follows: DL-isoproterenol, 491%; acetylcholine, 203%; corticotropin, 330%; EGTA, 41%; glucagon + EGTA, 375%; glucagon, 130%; ouabain, 214%; prostaglandin $E_1$, 825%; TSH, 134%. The extracellular levels of cyclic AMP from the same islet incubation were also measured (Table 12). Some parallelism between the intra- and extra-cellular cyclic AMP levels as influenced by various agents was noted.

## Separation of Cyclic AMP from Other Nucleotides

The column technique (Table 13) utilized was slightly different from that of Krishna et al. (38). Theophylline was separated so well from cyclic AMP that it could not in-

Fig. 13. Standard curve for the measurement of cyclic AMP with a partially purified preparation of bovine heart cyclic AMP dependent protein kinase. Assay conditions were as described under "Experimental Procedure". The activity of the heart enzyme was proportional to cyclic AMP amount within 10 pmoles.
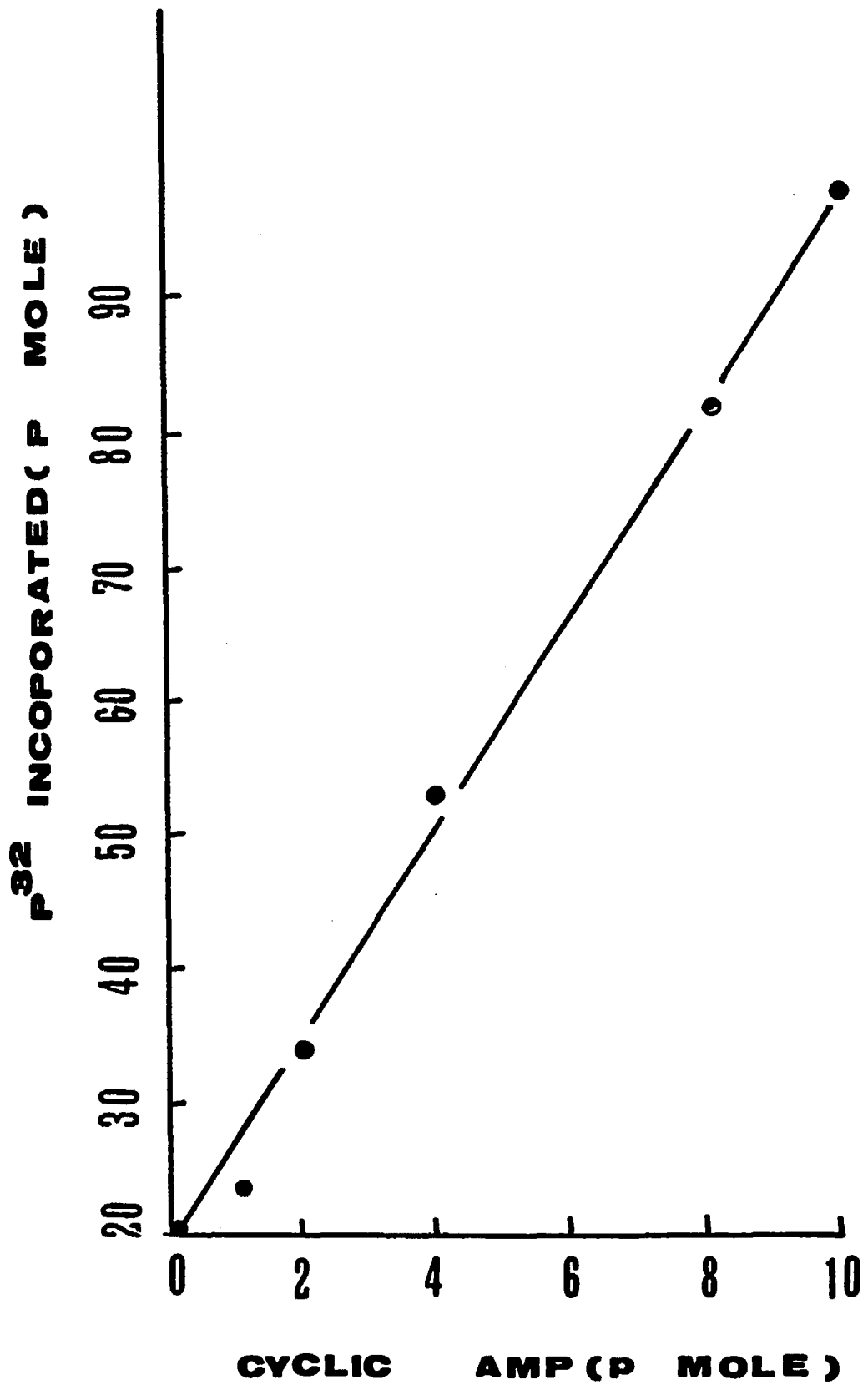
Fig. 13 - Standard curve for the measurement of cyclic AMP with cyclic AMP dependent protein kinase.

Table 12. Effects of various agents on cyclic AMP levels in pancreatic
islets. Isolation and incubation of islets and measurements of the amount of
total cyclic AMP by the protein kinase catalytic method were as described under
"Experimental Procedure". About 225 islets were present in each incubation tube
of 5 ml Krebs-Ringer bicarbonate buffer containing 10 mM theophylline. Each tube
was incubated for 10 min at 37° in a shaking water bath. Each incubation was per-
formed in duplicate and the mean is presented. Each sample from the individual
incubations was assayed for cyclic AMP in triplicate. Values presented in the
parentheses represent the means of such triplicate assays.

[a]Absolute level found in islets after 10 min incubation.

[b]Values corrected for cyclic AMP level found in islets before incubation
(zero time control), which amounted to 49.0 pmoles/mg protein.

[c]Absolute level found in the incubation medium (free of islets) after 10
min incubation.

## TABLE 12

## EFFECTS OF VARIOUS AGENTS ON CYCLIC AMP LEVELS IN PANCREATIC ISLETS

| Agent and concentration | Intracellular cyclic AMP | | Extracellular cyclic AMP (total)[c] |
| --- | --- | --- | --- |
| | Total[a] | Change[b] | |
| | pmoles/mg islet protein | | pmoles/mg islet protein |
| None (control) | 61.9 (60.0;63.7) | 12.9 | 4.4 |
| DL-Isoproterenol (0.1 mM) | 125.3 (123.4;127.2) | 76.3 | 11.2 |
| Acetylcholine (0.1 mM) | 88.1 (82.8;93.4) | 39.1 | 13.3 |
| Corticotropin (0.05 mM) | 91.6 (89.7;98.4) | 42.6 | 8.9 |
| Glucagon (0.01 mM) | 78.7 (74.9;82.4) | 29.7 | 6.9 |
| Thyroid-stimulating hormone (20 μg/ml) | 79.2 (76.0;82.4) | 30.2 | 8.9 |
| Prostaglandin $E_1$ (20 μg/ml) | 118.4 (158.7;178.0) | 119.4 | 14.1 |
| EGTA (2 mM) | 67.3 (67.3;67.3) | 18.3 | 6.8 |
| Glucagon (0.01 mM) + EGTA (2 mM) | 110.4 (108.5;112.3) | 61.4 | 3.4 |

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## TABLE 13

### PURIFICATION OF CYCLIC AMP WITH AG-50WX8 COLUMNS
### (0.5 X 2.5 cm)

| mls of eluate collected | nucleotides, etc. |
|---|---|
| 0.5 ml of sample loaded + first 2 ml of eluate | ATP, ADP, Theophylline |
| 3rd and 4th ml | Cyclic AMP |

0.5 ml of samples were loaded, and eluted with water. 5'-AMP did not appear in the first 4 mls of collected eluate. The separation between different nucleotides was identified by optical density (260 mμ) and radioactive counts of ATP and cyclic AMP.

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TABLE 14

MIGRATION OF SOME ADENOSINE NUCLEOTIDES ON SILICAR
TLC-7GF MALLINCKRODT

| Compound | $R_F$ |
|----------|-------|
| Cyclic AMP | 0.91 |
| 5'-AMP | 0.55 |
| ADP | 0.18 |
| ATP | 0.14 |

Developed with isopropyl alcohol-water-29% $NH_4OH$
(7:2:1, v/v) as solvent for 5 hours. All spots were checked
under ultraviolet light.

terfere with the cyclic AMP recovery examined by optical density (260 m$\mu$) in adenylate cyclase assay.

In addition to the observation of $R_f$ values of cyclic AMP and 5'-AMP (77), the $R_f$ values of both ATP and ADP were examined with SilicAR TLC-7GF Mallincrodt (Table 14), and the results confirmed that the thin layer chromatography technique was good enough to examine the purity of cyclic AMP in adenylate cyclase assay.

CHAPTER IV

DISCUSSION

The pancreatic islet isolation procedure is a mo-
dified version of that developed by Lacy and Kostianovsky
(20). The most significant innovation in the present pro-
cedure is the use of pilocarpine, a potent cholinomimetic
agent. This drug has been shown to cause a complete or a
near-complete depletion of zymogen granules from pancre-
atic acinar tissue (78). These zymogens, if present, could
potentially be released and activated during the collagen-
ase digestion of pancreatic tissue. The active enzymes,
especially the proteolytic ones, could interfere with the
isolation procedure and destroy the islet plasma membrane
structure.

The use of a 180-mesh silk screen to retain islets
during the washing of the collagenase-digested pancreatic
tissue and the use of an 18-gauge needle attached to a
siphon device for picking the islets greatly facilitate
the isolation of islets in adequate amounts for the experi-
ments. The islets obtained by this procedure are largely

free of acinar tissue as judged by a microscopic exami-
nation. The islet preparations, containing 1.7 $\pm$ 0.1 $\mu$g
protein per islet, compare favorably with the value of
about 3 $\mu$g protein per islet reported by others (44).
Although the islet preparations are undoubtedly conta-
minated with small amounts of exocrine pancreatic tissue,
the extent to which this contributed to the total adeny-
late cyclase activity may be minimal. The $\beta$-cells, which
are actually involved in insulin secretion, comprise
about 60-90% of the islet cell content (79). The res-
ponses of islet adenylate cyclase to many hormones and
agents, might also largely represent those of $\beta$-cell
adenylate cyclase.

The advantages of the pilocarpine pretreatment of
rats prior to preparation of the islets are clear. First,
it permits an increased yield of islets. Second, adenylate
cyclase in the isolated islets, perhaps with its receptors
remaining relatively undamaged, retains its responsiveness
to a wide variety of hormones. Author has observed with is-
lets obtained from rats not pretreated with pilocarpine
that adenylate cyclase activity shows little or no res-
ponse to glucagon, whereas, under the same experimental
conditions, this hormone causes a doubling of the enzyme
activity in islets obtained from pretreated rats. These
results may be somewhat analogous to the f ndings of Rod-
bell et al. made with fat cell ghosts (45), wherein tryp-

sin digestion of the plasma membrane causes a loss in the sensitivity of adenylate cyclase to hormones. It is also reasonable to assume that cholinergic stimulation leads to the discharge of insulin (and possibly glucagon) from the islet tissue. Acetylcholine or vagal stimulation have been shown to initiate insulin secretion (60, 80). Pre-treatment with pilocarpine could thus increase the responsiveness of islet adenylate cyclase by decreasing endogenous insulin or glucagon in the tissue preparation. This step also helped to assure that the author was not observing the combined effects of endogenous polypeptides and the various test substances investigated. The results of the present study indicate that islet adenylate cyclase is responsive to a great number of stimulants, perhaps reflecting a central role of insulin in many homeostatic mechanisms. In a recently published communication, Rosen et al. described a study concerning adenylate cyclase activity of an insulin-producing islet cell tumor (83). They reported that only glucagon and fluoride, among many substances tested, were capable of stimulating islet tumor adenylate cyclase. The agents tested and found to be ineffective were: epinephrine, norepinephrine, isoproterenol, arginine, glucose, prostaglandin $E_1$, ethanol, secretin, insulin and ACTH. Their results are obviously at variance with the findings. It should be emphasized, however, that the adenylate cyclase

which the author studied is from normal rats which had been
treated with pilocarpine. The purity of some hormones, such
as pancreozymin, secretin, and TSH, used in the present
study is rather low. The effects seen with these hormone
preparations remain to be confirmed when pure hormones be-
come available to the author.

It has been shown (60, 61, 80) that acetylcholine
and vagal stimulation cause an increase in insulin secre-
tion. (The author has also observed that acetylcholine el-
evates cyclic AMP levels in the intact pancreatic islets
prepared from rats pretreated with pilocarpine.) This effect
of acetylcholine on insulin secretion may be an indirect one.
It may be explained, at least in part, by assuming that
acetylcholine interacts with islet $\alpha$-cells and leads to a
release of glucagon. The glucagon, in turn, activates the
adenylate cyclase of neighboring $\beta$-cells, resulting in
elevated cellular cyclic AMP levels. The effect on pancre-
atic islet cyclic AMP production by both insulin and glu-
cagon has been confirmed, but the major target cells of
these two hormones is not absolutely clear. The finding
that acetylcholine stimulates adenylate cyclase in the is-
let homogenates (Table 4) is quite unexpected. Nevertheless,
it provides direct evidence supporting the contention that
the action of acetylcholine on the organelles (and perhaps
$\beta$-cells) may be a direct one. The ability of acetylcholine,
like many hormones, to activate islet adenylate cyclase may

be of particular physiological significance. It remains to be seen whether acetylcholine also increases cyclic GMP levels in islets. Several lines of recent evidence have suggested that cyclic GMP, but not cyclic AMP, may be involved in cholinergic neurotransmission and that acetylcholine functions to specifically increase the tissue levels of cyclic GMP (84-86).

All hormones, drugs, compounds and metabolites in this study were examined for their possible effects on islet phosphodiesterase assayed in the presence and absence of 10 mM theophylline. At concentrations the same as those employed in the adenylate cyclase studies, none of these hormones and agents had any significant effects on the phosphodiesterase activity[1]. Goldfine et al. (70) reported that islet cell tumor phosphodiesterase is inhibited by tolbutamide which has a $K_i$ of 3 mM. In their study, however, the concentrations of tolbutamide found to significantly inhibit phosphodiesterase were about an order of magnitude higher than those required to activate adenylate cyclase of normal islets as observed in the studies (Table 5) and in islet cell adenoma as reported by Levey et al. (69).

Most of the hormones and agents studied for their

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[1]Kuo, W. N., Hodgins, D. S., Kuo, J. F. Adenylate cyclase in islets of Langerhans, manuscript to be submitted for publication.

effects on adenylate cyclase in islet homogenates were
also examined for their effects on the cyclic AMP levels
in intact islets, employing either the technique of pre-
labeling the cellular ATP pool with radioactive adenine
(87, 88) or with the protein kinase catalytic method (33).
Most of the results obtained with the broken cell system
were confirmed with the intact cell experiments, except
for NaF which was not effective in increasing cyclic AMP
levels in intact islets. NaF was shown earlier to be a
potent stimulator of adenylate cyclase in fat cell ghosts
(89) while having no effect on intact fat cells (88). Be-
cause of the three different kinds of techniques employed
under different conditions, it was not necessary to get
exactly the same degree of effect on pancreatic islet cyclic
AMP production by various agents. Glucagon, isoproterenol,
prostaglandins ($E_1$, $E_2$, $A_1$, $F_{1\alpha}$), ACTH, secretin, acetyl-
choline, TSH, tolbutamide, EGTA, GTP, and epinephrine or
norepinephrine in the presence of $\alpha$-adrenergic blockers
were found to increase cyclic AMP level in rat pancreatic
islets. The increase in intracellular cyclic AMP levels
evoked by these agents were apparently due to their sti-
mulation of islet adenylate cyclase. In addition to the
above mentioned agents, pancreozymin, somatotropin, GDP,
ethanol and NaF were also found to stimulate islet adeny-
late cyclase. Many of these agents are known to have a

physiological role or pharmacological effect in promoting insulin secretion. Another class of insulinogenic agents, such as glucose, arginine, leucine, and phenformin (an oral hypoglycemic drug), whose action is considered to be mediated through an intracellular mechanism (62) did not increase islet cyclic AMP levels. They inhibited slightly or exerted no effect on islet adenylate cyclase. Many of the hormones studied with islets are also known to act on rat fat cells (36, 81, 82). The obvious differences seen in rat fat cells are that prostaglandins, particularly in the presence of catecholamines and other lipolytic hormones, decrease cyclic AMP levels (88, 92), and that epinephrine or norepinephrine (in the absence or presence of $\alpha$-adrenergic blockers) increase cyclic AMP levels (36, 81, 82), suggesting adrenergic receptors in rat fat cells are functionally of the $\beta$-type. It is worthy mentioning that, like rat islets, human fat cells were found to possess both $\alpha$-and $\beta$-adrenergic receptors (93). The acetylcholine-mediated stimulation of adenylate cyclase and increase of cyclic AMP levels in rat islets, to the author's knowledge, have not yet been found in other tissues. Cholinergic agents have been shown to stimulate the release of insulin from slices of rat pancreas (60, 61) and islets (40).

The author has not studied the effects of hormones and other agents on insulin secretion by the islet preparation, since pilocarpine pretreatment of rats may have

depleted the insulin stores of the $\beta$-cells.

Cyclic AMP, as has been previously hypothesized, appears to play an important role in the secretion, and possibly the synthesis, of insulin (for example, see 22, 23, 40, 42, 44). The exact mechanism for the action of cyclic AMP, however, remains unclear. Since a complete correlation between adenylate cyclase activation and the stimulation of insulin secretion appears to be lacking, intracellular cyclic AMP levels alone may not be the sole determinants of insulin secretion. It seems at least two separate secretory mechanisms are involved; one being cyclic AMP-mediated and the other being closely associated with the metabolism of glucose and certain amino acids. Another possibility is a mechanism that requires a combination of the effects of both cyclic AMP and metabolites. Thus, increased intracellular cyclic AMP levels may facilitate insulin secretion as elicited by metabolites. However, a cyclic AMP dependent protein kinase, and a protein kinase modulator activity have been found in islet[2]. The islet protein kinase has some catalytic properties common to the enzyme found in many other sources; these include the artificial substrate and cyclic nucleotide specificity,

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[2]Kuo, J. F., Kuo, W. N., Hodgins, D. S., Greengard, P. Cyclic AMP dependent protein kinase from islets of Langerhans, manuscript to be submitted for publication.

and the metal ion requirement. No cyclic GMP dependent protein kinase activity was found. It is reasonable to assume that the phosphorylation catalyzed by cyclic AMP dependent protein kinase inside the $\beta$-cells of islet tissue could lead to the decomposition of glycogen and triglyceride, the conformational change of histones, microtubular elements, and membrane proteins. Furthermore, all of above phosphorylation functions could ultimately facilitate both insulin biosynthesis and insulin secretion (Fig. 14). The modulator inhibits or activates cyclic AMP dependent and cyclic GMP dependent protein kinase under certain conditions (90, 91), suggesting a specific role for cyclic AMP in the secretion and possibly synthesis of insulin.

In addition to experiments using whole isolated islets it may be quite possible to study:(a) the cyclic AMP system in $\alpha$-cells of pancreatic islet tissue by alloxan pretreatment of rats to result in the selective damage of $\beta$-cells (94), (b) the secretion of insulinogenic hormones regulated directly or indirectly by insulin mediated through cyclic AMP system, (c) and the effect of gastrin, tetragastrin, glucagon-like hormone, serotonin, and some other analogs of cyclic AMP (tubercidin 3', 5'-monophosphate, 3'-methylene cyclic phosphonate analog, 5'-methylene cyclic phosphonate analog, and dibutyryl analog of cyclic AMP) on pancreatic islet cyclic AMP production.

Various agents ————→ Islet adenylate cyclase
                              (or islet phosphodiesterase)

————————————————— Cyclic AMP

Cyclic AMP dependent
protein kinase

Energy production          Energy production by lipid
by carbohydrate           oxidation, conformational
oxidation                    changes of proteins

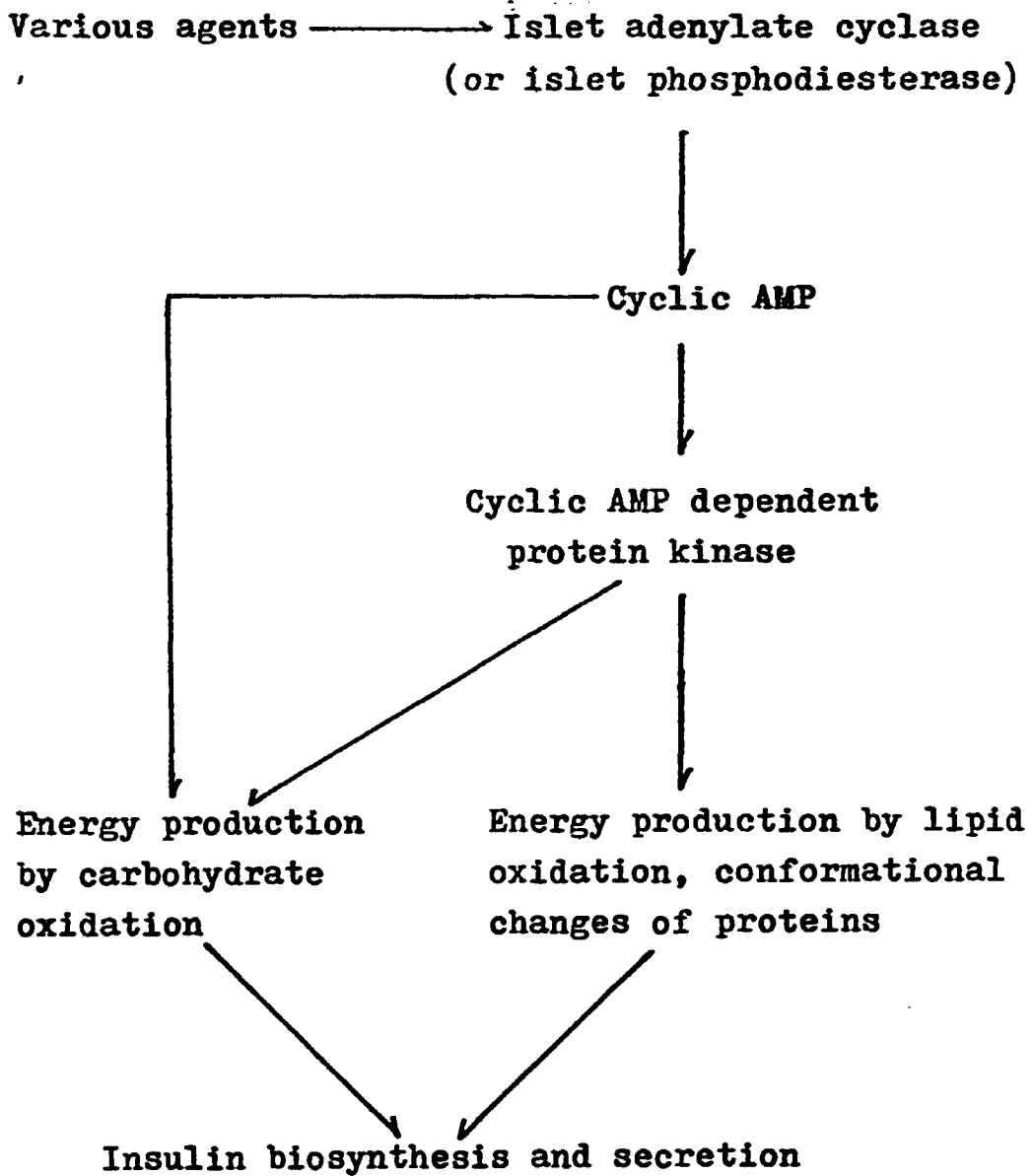Insulin biosynthesis and secretion

Fig. 14 - Possible mechanism of cyclic AMP
system on insulin secretion.

# CHAPTER V

## SUMMARY

An improved method for the isolation of rat pancreatic islets possessing adenylate cyclase responsive to various hormones is described, The key step in this method, which yielded up to 500 islets per pancreas, involved pretreatment of rats with pilocarpine. This allowed depletion of zymogens from the exocrine pancreatic tissue and thus minimized the destruction of islet membranal structure which could otherwise occur during the collagenase digestion of the tissue.

The adenylate cyclase activity in the islet homogenates was assayed in the presence of a variety of hormones and other agents. With the exception of some insulinogenic metabolites, such as goucose, arginine and leucine, all hormones previously demonstrated by others to stimulate insulin secretion were found to augment the synthesis of cyclic AMP by islet adenylate cyclase. These agents include glucagon, adrenocorticotropic hormone, secretin, pancreozymin, various prostaglandins, and $\beta$-adrenergic agents. Acetylcholine, thyrotropin, somatotropin, ethanol, EGTA, GTP, GDP, and NaF

also stimulated cyclic AMP production, but their insulino-
genic action has not been clearly demonstrated. Of the two
hypoglycemic drugs studied, tolbutamide was found to stimu-
late the cyclase activity whereas phenformin was without
effect. Insulin inhibited the islet adenylate cyclase acti-
vity, suggesting a possible feed-back mechanism involved.
None of these agents had any effect on the islet phospho-
diesterase activity.

Regulation by various agents of cyclic AMP levels
in intact islets of rat pancreas was studied, employing
either the technique of prelabeling the cellular ATP pool
or the protein kinase catalytic method. The results obtain-
ed with the intact cell system confirmed most of the find-
ings made with the broken cell system, clearly suggesting
a role for cyclic AMP in the action of certain insulino-
genic agents.

# BIBLIOGRAPHY

1. Sutherland, E. W., Rall, T. W. 1957 The properties of an adenine ribonucleotide produced with cellular particles, ATP, Mg$^{+}$ and epinephrine or glucagon, J. Am. Chem. Soc., 79 : 3608.

2. Sutherland, E. W., Rall, T. W. 1958 Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles, J. Biol. Chem., 232 : 1077.

3. Rall, T. W., Sutherland, E. W. 1958 Formation of a cyclic adenine ribonucleotide by tissue particles, J. Biol. Chem., 232 : 1065.

4. Sutherland, E. W. 1965 The action of epinephrine and the role of the adenyl cyclase system in hormone action, Recent Progr. Hormone Res., 21 : 623.

5. Butcher, R. W., Sutherland, E. W. 1962 Adenosine 3', 5'-phosphate in biological meterials, J. Biol. Chem., 237 : 1244.

6. Rall, T. W., Sutherland, E. W., Berthet, J. 1957 The regulation of epinephrine and glucagon to liver phosphorylase, J. Biol. Chem., 224 : 463.

7. Sutherland, E. W., Rall, T. W. 1960 The regulation of

adenosine 3', 5'-phosphate and phosphorylase to the actions of catecholamines and other hormones, Pharmacol. Rev., 12 : 265.

8. Robison, G. A., Butcher, R. W., Sutherland, E. W. 1967 Adenyl cyclase as an adrenergic receptor, Ann. N. Y. Acad. Sci., 139 : 703.

9. Robison, G. A., Butcher, R. W., Sutherland. E. W. 1971 Cyclic AMP. Academic Press, New York and London.

10. Sutherland, E. W., Robison, G. A., Butcher, R. W. 1968 Some aspects of the biological role of cyclic AMP, Circulation 37 : 279.

11. Kuo, J. F., Greengard, P. 1969 An adenosin 3', 5'-monophosphate dependent protein kinase from E. coli, J. Biol. Chem., 224 : 3417.

12. Kuo, J. F., Greengard, P. 1969 Widespred occurence of adenosine 3', 5'-monophosphate dependent protein kinase in various tissues and phyla of the animal kingdom. Proc. Nat. Acad. Sci. USA, 64 : 1349.

13. Kuo, J. F., Greengard, P. 1970 Isolation and partial purification of a protein kinase activated by guanosine 3', 5'-monophosphate, J. Biol. Chem., 245 : 2493.

14. Langan, T. A. 1969 Action of adenosine 3', 5'-monophosphate dependent histone kinase in vivo, J. Biol. Chem., 244 : 5763.

15. Corbin, J. D., Reiman, E. M., Walsh, D. A., Kreb, E. G.

1970 Activation of adipose tissue lipase by ske-
letal muscle cyclic adenosine 3', 5'-monophosphate
-stimulated protein kinase, J. Biol. Chem., 245 :
4849.

16. Goodman, D. B. P., Rasmussen, H., Dibella, F. 1970
Cyclic adenosine 3', 5'-monophosphate stimulated
phosphorylation of isolated neurotubule subunits,
Proc. Nat. Acad. Sci. U. S., 67 : 652.

17. Delange, R. J., Kemp, R. G., Riley, W. D., Copper, R.
A., Krebs, E. G. 1968 Activation of sketal muscle
phosphorylase kinase by adenosine triphosphate
and adenosine 3', 5'-monophosphate, J. Biol. Chem.,
243 : 2200.

18. Walsh, D. A., Perkins, J. P., Krebs, E. G. 1968 An
adenosine 3', 5'-monophosphate dependent protein
kinase from rabbit skeletal muscle, J. Biol. Chem.,
243 : 3763.

19. Schlender, K. K., Wei, S. H., Villar-Palasi, C. 1969
UDP-glucose : glycogen ⍺-4-Glucosyltransferase I
kinase activity of purified muscle protein kinase
cyclic nucleotide specificity, Biochim. Biophy.
Acta, 191 : 272.

20. Lacy, P. E., Kostianovsky, M. 1967 Method for the iso-
lation of intact islets of Langerhans from the rat
pancreas, Diabetes, 16 : 35.

21. Mayhew, D. A., Wright, P. H., Ashmore, J. 1969 Regu-
lation of insulin secretion, Pharmacological Re-
views, 21 : 183.

22. Sussman, K. E., Vaughn, G. D. 1967 Insulin release after ACTH, glucagon and cyclic AMP in the perfused isolated rat pancreas, Diabetes, 16 : 449.

23. Turtle, J. R., Kipnis, D. A. 1967 An adrenergic receptor mechanism for the control of cyclic 3', 5'-adenosine monophosphate synthesis in tissues, Biochemical and Biophysical Reseasch Communication, 28 : 797.

24. Ashcroft, S. J. H., Randle, P. J. 1968 Glucose-6-phosphatase activity of mouse pancreatic islets, Nature, 219 : 857.

25. Brolin, S. E., Berne, C. 1967 The enzymatic activities of the initial glycolytic steps in pancreatic islets and acini, Metaleolism, 16 : 1024.

26. Lazarow, A. 1963 Functional characterization and metabolic pathways of the pancreatic islet tissue, Recent Progr. Hormone Res., 19 : 489.

27. Taljedal, I. B. 1967 Electrophoretic studies on phosphatases from the pancreatic islets of obese-hyperglycemic mice, Acta Endocrinol., 55 : 153.

28. Taljedal, I. B. 1966 Electrophoretic separation of enzymes from mammalian pancreatic islets, Med. Pharmacol. Exp. 15 : 603.

29. Taljedal, J. S. 1968 Stimulation of 5-nucleotides activity in the pancreatic islets of rats by glucagon, Endocrinology 82 : 173.

30. Robison, G. A., Butcher, D. W., Sutherland, E. W. 1968 Cyclic AMP, Rev. Biochem., 37 : 149.

31. Coore, H. G., Randle, P. J. 1964 Regulation of insulin secretion studied with pieces of rabbit pancreas incubated _in vitro_, Biochem. J., 93 : 66.

32. Malaisse, W., Malaisse-Lagae, F., Wright, P. A. 1967 A new method for the measurement _in vitro_ of pancreatic insulin secretion, Endocrinology, 80 : 99.

33. Kuo, J. F., and Greengard, P. 1970 An assay method for the measurement of adenosine 3', 5'-monophosphate in various tissues and a study of agents influencing its level in adipose cells, J. Biol. Chem., 245 : 4067.

34. Post, R. L., Sen, A. K. 1967 _Methods in Enzymology_, Vol X, Academic Press, New York, P. 773.

35. Hanks, J. H. 1948 The longevity of chick tissue cultures without renewal of medium, J. Cellular Comp. Physiol., 31 : 235.

36. Rodbell, M., Birnbaumer, L., Pohl, S. L. 1970 Adenyl cyclase fat cells, J. Biol. Chem., 245 : 718.

37. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951 Protein measurement with the folin phenol reagent, J. Biol. Chem., 193 : 265.

38. Krishna, G., Weiss, B., Brodie, B. B. 1968 A simple ,

sensitive method for the assay of adenyl cyclase,
J. Pharm. Exp. Therap., 163 : 379.

39.  Gardner, R. S., Wahba, A. J., Basilio, C., Miller, R.
     S., Lengyel, P., Speyer, J. F. 1962 Synthetic po-
     lynucleotides and the amino acid code, Proc. Nat.
     Acad. Sci. U. S., 48 : 2087.

40.  Vance, J. E., Buchanan, K. D., Williams, R. H. 1971
     Glucagon and insulin release Diabetes, 20 : 78.

41.  Devrim, S., Recant, L. 1966 Effect of glucagon on in-
     sulin release in vitro, Lancet, 2 : 1227.

42.  Malaisse, W. J., Malaisse-Lagae, F., Mahew, D. 1967
     A possible role for the adenylcyclase system in
     insulin secretion, J. Clin. Invest., 46 : 1724.

43.  Birnbaumer, L., Rodbell, M. 1969 Adenyl cyclase in
     fat cells, J. Biol. Chem., 244 : 3477.

44.  Montague, W., Cook, J. R. 1971 The role of adenosine
     3':5'-cyclic monophosphate in regulation of in-
     sulin release by isolated rat islets of Langer-
     hans, Biochem. J., 122 : 115.

45.  Rodbell, M., Birnbaumer, L., Pohl, S. L., Krans, H.
     M. J. 1971 The glucagon-sensitive adenyl cyclase
     system plasma membrane of rat liver, J. Biol.
     Chem., 246 : 1877.

46.  Rodbell, M., Krans, H. M. J., Pohl, S. L., Birnbaumer,
     L. 1971 The glucagon-sensitive adenyl cyclase sys-

tem plasma membrane of rat liver, J. Biol. Chem.,
<u>246</u> : 1872.

47. Hepp, K. D., Edel, R., Wieland, O. 1970 The effects
of glucagon and fluoride on a particular prepara-
tion from rat and mouse liver, Eur. J. Biochem.,
<u>17</u> : 171.

48. Pfeiffer, E. F., Telib, M., Ammon, J., Melani, F.,
Ditschuneit, H. 1966 The direct stimulation by
secretin of insulin secretion <u>in vitro</u>, Ger.
Med. Monthly, <u>11</u> : 432.

49. Telib, M., Pfeiffer, G. F. 1970 Dynamic of insulin
secretion <u>in vitro</u>:effects of repeated induction
of insulin release by single and multiple stimu-
lants, Endokrin., <u>56</u> : 189.

50. Buchanan, K. D., Vance, J. E., William, R. H. 1969
Insulin and glucagon release from isolated is-
lets of Langerhans, Diabetes, <u>18</u> : 381.

51. Lazarus, S. S., Shapiro, S., Volk, B. W. 1968 Secret-
ory granule formation and release in rabbit pan-
creatic A-cells, Diabetes, <u>17</u> : 152.

52. Lazarus, N. R., Voyles, N. R., Tanese, T., Devrim,
S., Recant, L. 1968 Extra-gastrointestinal effects
of secretin, gastrin, and pancreozymin, Lancet,
<u>2</u> : 248.

53. Lobovitz, H. E., Pooler, K. 1967 ACTH-mediated in-
sulin secretion:effect of aminophylline, Endo-

crinology, 81 : 558.

54. Yalow, R. S., Black, H., Villazon, M., Berson, S. A.
    1960 Comparison of plasma insulin levels follow-
    ing administration of tolbutamide and glucose,
    Diabetes, 9 : 356.

55. Malaisse, W. J., Malaisse-Lagae, F., Kind, S., Wright,
    P. H. 1968 Effect of growth hormone on insulin
    secretion, Amer, J. Physiol., 215 : 423.

56. Milner, R. D. 1970 The stimulation of insulin release
    by essential amino acids from rabbit pancreas in
    vitro, J. Endocrim., 47 : 247.

57. Gorman, R. E., Bitensky, M. W. 1970 Selective activa-
    tion by short chain alcohols of glucagon respon-
    sive adenyl cyclase in liver, Endocrinology, 87 :
    1075.

58. Metz, R., Berger, S., Mako, M. 1969 Potentiation of
    the plasma insulin response to glucose by prior
    administration of alcohol, Diabetes, 18 : 517.

59. Phillips, G. B., Safrit, H. F. 1971 Alcholic diabetes,
    J. Amer. Med. Assoc., 217 : 1513.

60. Mahew, D. A., Goldberg, A. M., Wright, P. H. 1968 The
    influence of cholinomimetics upon insulin secre-
    tion, Diabetes, 17 : 308.

61. Malaisse, W. J., Malaisse-Lagae, F., Wright, P. H.,
    Ashmore, J. 1967 Effects of adrenergic and cho-

linergic agents upon insulin secretion in vitro, Endocrinology, 80 : 975.

62. Atkins, T., Matty, A. J. 1971 Adenyl cyclase and phosphodiesterase activity in the isolated islets of Langerhans of obese mice and their lean litter mates: the effect of glucose, adrenaline and drugs on adenyl cyclase activity, J. Endocrin., 51 : 67.

63. Ashcroft, S. J. H., and Randle, P. J. 1968 Glucose metabolism and insulin release by pancreatic islets, Lancet, 1 : 278.

64. Ashcroft, S. J. H., Coll-Garcia, E., Gill, J. R., Randle, P. J. 1968 Glucose metabolism, islets and insulin release, Diabetologia, 4 : 178.

65. Howell, S. L., Taylor, K. W. 1968 Potassium ions and the secretion of insulin by islets of Langerhans incubated in vitro, Biochem. J., 108 : 17.

66. Selzer, H. S. 1962 Quantitative effects of glucose, sulfonylureas, salicylate, and indole-3-acetic acid on the secretion of insulin activity into pancreatic venous blood, J. Clin. Invest., 41 : 289.

67. Yu, T., Berger, L. Gutman, A. B. 1968 Hypoglycemic and uricosuric properties of acetohexamide and hydroxyhexamide, Metab. Clin. Exptl., 17 : 309.

68. Levey, G. S., Palmer, R. F. Lasser, K. C., McCarthy, J. 1971 Effect of tolbutamide on adenyl cyclase

in rabbit and human heart and contractility of
isolated rabbit atria, J. Clin. Endocrin., 33 :
371.

69. Levey, G. S., Schmidt, W. M. I., Mintz, D. H. 1972
Activation of adenyl cyclase in pancreatic is-
let cell adenoma by glucagon and tobutamide,
Metabolism, 21 : 93.

70. Goldfine, I. D., Pearlman, R. T., Roth, J. 1971 In-
hibition of cyclic 3', 5'-AMP phosphodiesterase
in islet cells and other tissues by tobutamide,
Nature, 234 : 295.

71. Brown, J. D., Steels, A. A., Stone, D. B., Steel, F.
A. 1972 The effect of tobutamide on lipolysis
and cyclic AMP concentration in white fat cells,
Endocrinology, 90 : 47.

72. Hellman, B., Sehlin, J., Täljedal, I.-B. 1971 The
pancreatic β-cell recognition of insulin sec-
retagogues, Biochem. Biophys. Res. Commun., 45 :
1384.

73. Macleod, A. G. 1967 Diabetes. The Upjohn Co., Kala-
mazoo, Mich., P. 84.

74. Bressler, R., Vargas-Cordon, M., Lebovitz, H. E. 1968
Tranylcypromine: a potent insulin secretagogue
and hypoglycemic agent, Diabetes, 17 : 617.

75. Feldman, J. M., Lebovitz, H. E. 1970 Mechanism of
epinephrine and serotonin inhibition of insulin
release in the golden hamster in vitro, Diabetes,

__19__ : 480.

76. Porte,D. 1967 Beta adrenergic stimulation of insulin release in man, Diabetes, __16__ : 150.

77. Goldberg, N. D., Dietz, S. B., O'Toole, A. G. 1969 Cyclic GMP in mammalian tissue and urine, J. Biol. Chem. __224__ : 4458.

78. Covell, W. P. 1928 A microscopic study of pancreatic secretion in the living animal, Anat. Rec., __40__ : 213.

79. Hartcroft, W. S. 1956 Islet pathology in diabetes, Diabetes, __5__ : 98.

80. Kizer, J. S., Bressler, R. 1969 Drugs and the mechanism of insulin secretion, Adv. in Pharmacology and Exp. Chemotherapy, __7__ : 91.

81. Bär, H. P., Hechtor, O. 1969 Adenyl cyclase and hormone action, Proc. Nat. Acad. Sci. U. S., __63__ : 350.

82. Kuo, J. F. 1970 Differential effects of $Ca^{++}$, EDTA and adrenergic blocking agents on the actions of some hormones on cyclic AMP levels in isolated adipose cells as determined by prior labeling with $[8-^{14}C]$ adenine, Biochim Biopys. Acta, __208__ : 509.

83. Rosen, O. M., Hirsh, A. H., Goren, E. N. 1971 Factors which influence cyclic AMP formation and de-

gradation in an islet cell tumor of the Syrian
hamster, Arch. Biochem. Biophys., $\underline{146}$ : 660.

84. George, W. J., Polson, J. B., O'Toole, A. G., Gold-
berg, N. D. 1970 Elevation of guanosine 3', 5'-
cyclic phosphate in rat heart after perfusion
with acetylcoline, Proc. Nat. Acad. Sci. USA,
$\underline{66}$ : 398.

85. Ferrendelli, J. A., Steiner, A. L., McDougal, D. R.,
Kipnis, D. M. 1970 The effect of oxotremorine
and atropine on cGMP and cAMP levels in mouse
cerebral cortex and cerebellum, Bipchem. Bio-
phys. Res. Commun., $\underline{41}$ : 1061.

86. Kuo, J. F., Lee, T. P., Reyes, P. L. Walton, K. G.,
Donnelly, T. E., Greengard, P. 1972 An assay
method for the measurement of guanosine 3', 5'-
monophosphate in various biological materials
and a study of agents regulating its levels in
heart and brain, J. Biol. Chem., $\underline{247}$ : 16.

87. Kuo, J. F. Dill. I. K. 1968 Antilipolytic action of
valinomycin and nonactin in isolated adipose
cells through inhibition of adenyl cyclase, Bio-
chem. Biophys. Res. Commun., $\underline{32}$ : 333.

88. Kuo, J. F., DeRenzo, E. C. 1969 A comparison of the
effects of lipolytic and antilipolytic agents
on adenosine 3', 5'-monophosphate levels in adi-
pose cells as determined by prior labeling with
adenine-8-$^{14}$C, J. Biol. Chem., $\underline{224}$ : 2252.

89. Rodbell, M. 1967 Metabolism of isolated fat cells,

J. Biol. Chem., <u>242</u> : 5744.

90. Kuo, J. F., Greengard, P. 1971 Stimulation of cyclic GMP dependent protein kinase by a protein fraction which inhibits cyclic AMP dependent protein kinases, Federation Proc., <u>30</u> : 1089.

91. Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E., Greengard, P. 1972 Protein kinase modulator-purification and properties, Federation Proc., <u>31</u> : 439.

92. Butcher, R. W., Baird, C. E. 1968 Effects of prostaglandins on adenosine 3', 5'-monophosphate levels in fat and other tissues, J. Biol. Chem., <u>243</u> : 1713.

93. Burns, T. W., Lagley, P. E. 1970 Lipolysis by human adipose tissue: the role of cyclic 3', 5'-adenosine monophosphate and adrenergic receptor sites, J. Lab. Clin. Med., <u>75</u> : 983.

94. Macleod, A. G. 1969 <u>Diabetes</u>. The Upjohn Co., Kalamazoo, Mich., P. 96.