STUDIES RELATING THE ACTION OF ISOPROTERENOL AND THE ADENYL CYCLASE SYSTEM IN RAT SALIVARY GLANDS

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THESIS

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF OTTAWA

MAY 1973

ACKNOWLEDGEMENTS

The author wishes to express her deepest gratitude to Dr. A. D'Iorio for his wise counselling and great patience during the course of this work.

She acknowledges with thanks the valuable criticisms and stimulating discussions provided by Dr. A. P. Gaunce, Dr. M. Muirhead, Dr. J. Diaz Borges, and other graduate students.

Sincere appreciation is expressed to Mrs. P. Peterkin, Mrs. B. Betz and Dr. R. Labow for their valuable help in the setting up of some of the experiments.

The receipt of an Ontario Graduate Fellowship for the first two years of the work, and the financial support of The Medical Research Council of Canada for the final two years (through a grant to Dr. A. D'Iorio) are both gratefully acknowledged.

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LIST OF ABBREVIATIONS

ATP	-	adenosine triphosphate
approx.	-	approximately
b.w.b.	-	boiling water bath
c.p.m.	-	counts per minute
cyclic AMP	-	adenosine 3':5'-cyclic monophosphate
COMT	-	catechol O-methyl transferase
DEAE	-	diethylaminoethyl
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease
d.p.m.	-	disintegrations per minute
EDTA	-	ethylenediaminetetraacetate
EGTA	-	[ethylenebis (oxyethylenenitrilo) tetraacetic acid]
i.p.	-	intraperitoneal
PEI	-	polyethyleneimino
PEP	-	phosphoenolpyruvate
r.t.	-	room temperature
SAM	-	S-adenosylmethionine
TCA	-	trichloroacetic acid
TLC	-	thin layer chromatography
RNA	-	ribonucleic acid
RNase	-	ribonuclease
u.v.	-	ultraviolet

INTRODUCTION

The two main salivary glands, the parotid and the submandibular, have become increasingly important as models in which to study the effects of catecholamines on β-adrenergic receptors. The short-term effects involve the excretion, resynthesis and storage of salivary enzymes. The rat parotid, with its uniquely high production of α-amylase is ideal for studying these functions. The long-term effects include hypertrophy of the gland and hyperplasia produced by chronic stimulation of β-adrenergic receptors. Isoproterenol is used in these studies because it is the prototype of compounds which act mainly at β-adrenergic receptors.

In other tissues, the β-adrenergic receptor was being equated to the adenyl cyclase system and this was believed to be the situation in salivary glands as well. The investigations described herein were initiated in an attempt to establish possible control mechanisms over the formation of cyclic AMP. The effects of many catecholamines on adenyl cyclase were to be studied *in vitro* and also changes in cyclase activity which might occur in the presence of inhibitors of catecholamine-catabolizing enzymes, etc. Unfortunately, absolute changes in adenyl cyclase activity are difficult to measure *in vitro* because the enzyme is membrane-bound and destroyed by homogenization. However, following *in vivo* injection of isoproterenol,

tissue levels of cyclic AMP were found to increase immediately and to decline rapidly within a few minutes. What was happening to the cyclic AMP? The purpose of the project was broadened to include the acceptor protein for cyclic AMP in these investigations, as well as control mechanisms over cyclic AMP levels in salivary glands. Two enzymes which were thought to be involved were protein kinase and catechol O-methyl transferase. Both were found to have high endogenous activity in parotid and submandibular glands. The subcellular distribution was determined for each enzyme and both were found to be present in the particulate fractions as well as the soluble fractions.

In order to assess whether protein kinase was involved in reactions coupling the second messenger, cyclic AMP, with various effector systems in the parotid cell, it was necessary to purify (partially) the enzyme and to determine its characteristics and mechanism of activation. Attempts were also made to identify natural substrates. One method of determining whether phosphorylation was related to the short-term or long-term events involved the actions of protein synthesis inhibitors. Actinomycin D had been shown by others to inhibit synthesis of a protein related to DNA synthesis during the first hour after isoproterenol, whereas puromycin could inhibit α-amylase formation indicating control of its synthesis at the translational level. Although some information was gained about the nature of endogenous substrates for protein kinase in parotid gland, the

problem is still unresolved.

Nevertheless, a great deal was learned about the activities of all three enzymes in salivary glands. Catechol O-methyl transferase may possibly exert control over the amount of catecholamines present in the tissue and hence over the degree of activation of adenyl cyclase and the amount of cyclic AMP formed. The amount of cyclic AMP formed is directly proportional to the amount of active protein kinase in the tissue. If precautions are taken to avoid the action of phosphatases, it can be shown that an injection of isoproterenol causes an increase in the intrinsic protein kinase activity in the tissue and that phosphorylation of tissue proteins by this kinase occurs in different subcellular fractions. The increases in phosphorylation of proteins can be related chronologically to functions known to take place in the main organelles of the fractions. Therefore, it is suggested that phosphorylation of another enzyme (or protein) may play a role in at least one of the sequence of steps between catecholamine binding at the β-adrenergic receptor and the varied responses of the parotid gland cell.

REVIEW OF THE LITERATURE

PART I: CATECHOLAMINES

A. ADRENERGIC RECEPTORS

1. Definitions

The efferent branches of the autonomic nervous system are designated as adrenergic or cholinergic depending on whether noradrenaline or acetylcholine is present as the neurotransmitter at the nerve ending. Acetylcholine is the general transmitting agent at all synapses in ganglia transmitting signals to tissues, as well as at parasympathetic nerve endings. Noradrenaline is the neurotransmitter at sympathetic nerve endings.

When one of these chemical transmitters is released from its storage vesicles in the nerve ending, it diffuses across a very narrow "synaptic cleft" to act on "receptor" sites (Figure 1) located in the effector cell. The hormone, adrenaline, released into the blood from the adrenal medulla, diffuses into the extracellular fluid and acts at the same "receptor" sites on effector cells of its target organ.

Adrenaline and noradrenaline are the naturally-occurring biogenic amines of a class of compounds known as catecholamines.

Figure 1: Target cell receptor site.

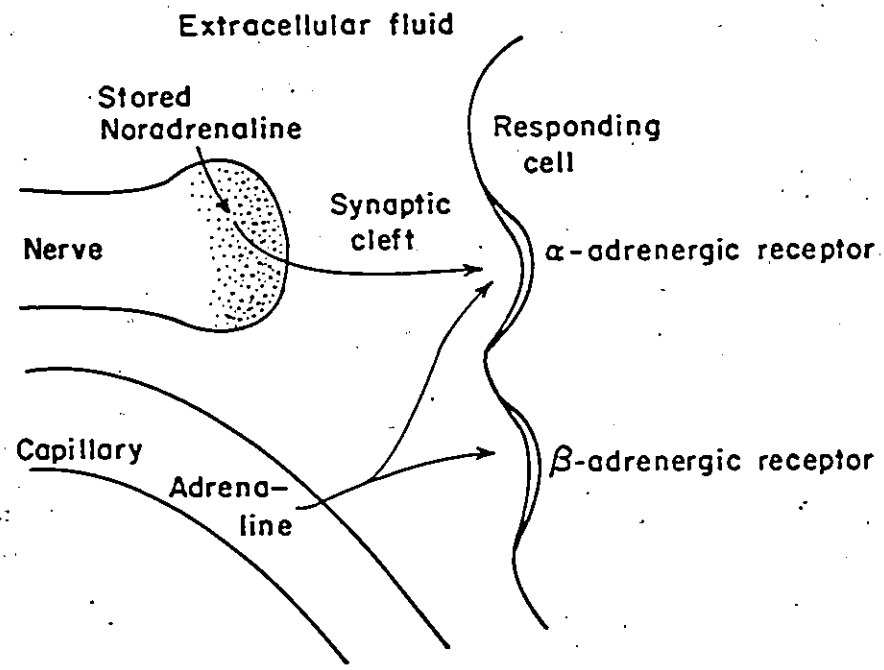
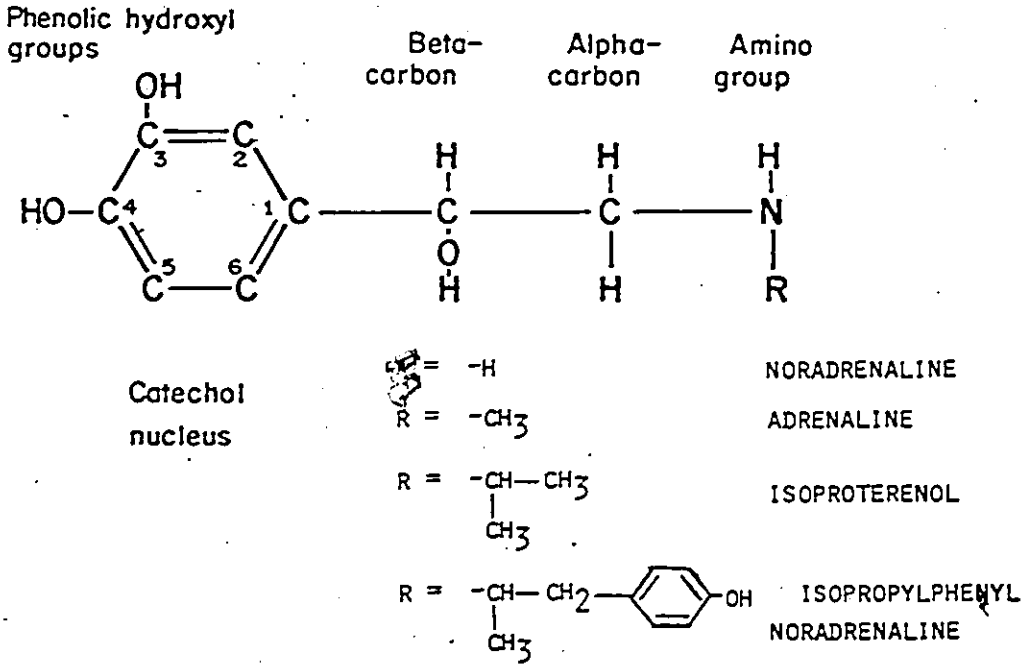


Figure 2: Catecholamine Structure.



Catecholamines are low molecular weight substances which contain a catechol nucleus and an amine group (Figure 2). Many other compounds, derivatives with the same basic structure, act, with varying degrees of potency, to produce the same effects at "adrenergic receptors".

Until recently adrenergic receptors were considered to be hypothetical structures located on muscle or gland cells and acted on by catecholamines to initiate or modify intracellular events resulting eventually in the observable response. Biochemical studies are bringing about, gradually, an understanding of adrenergic mechanisms. Receptors are now considered to be analogous to active sites or regulatory sites on an enzyme. The specific binding sites are a consequence of the amino acid sequence of the protein structure and the amino acid side chains present at the active site provide for both cybernetic and energetic coupling. First the active site must "recognize" the three-dimensional form of the catecholamine, then the nature of the binding forces between the catecholamine and the receptor protein will determine the affinity. The binding forces can take the form of covalent bonds, electrostatic forces, ion dipoles, hydrogen bonds or hydrophobic interactions. An affinity for the receptor is not enough to elicit a physiological response. A competitive inhibitor could act as a very effective blocking agent. The applicability of kinetic treatment to the action of hormones and receptors

has repeatedly been shown. Thus, according to the law of mass action, hormones react with receptors to yield hormone-receptor complexes. The dissociation constants (K_A) are equal to the hormone concentrations giving half-maximal effects and the affinity for a receptor then is given by $-\log K_A$.

The formation of the hormone-receptor complex can be considered as the initial step of a chain of events ultimately leading to a response:

$$H + R \rightleftharpoons HR \longrightarrow A \longrightarrow B \longrightarrow C \longrightarrow n \longrightarrow \text{response}$$

in which H symbolizes the neurohumoral agent and R the receptor. HR represents the complex of humoral agent and receptor. The formation of the complex may produce conformational changes in the protein; it may, in fact, be the modifier at the allosteric site of an enzyme. The reaction product will then instigate the sequence of biochemical events depicted by A, B, C and n leading to the measurable response, such as change of heart rate, oxygen consumption, altered enzyme activity, etc.

If we knew the precise coupling reactions of hormone and receptor in even one type of system this would be important in understanding related cases of intercellular communication. Unfortunately, the three-dimensional structure has not been determined yet for any receptor; in the meantime

the concept of "receptor" will continue to prove very useful, as it has in the past.

2. History of receptors

As early as 1905 the inhibitory effects of atropine on the actions of pilocarpine were interpreted by Langley as a competition for some "receptor substance"; however, Dale was the first to use the term in connection with the sympathetic nervous system in 1906. He demonstrated that certain ergot alkaloids were capable of blocking the excitatory but not the inhibitory actions of adrenaline so he postulated the existence of different receptive mechanisms for adrenaline. The classification of adrenergic receptors as alpha and beta was proposed by Ahlquist in 1948. The concept was based on the relative responsiveness of a series of catecholamines in producing specific physiological effects. Alpha receptors were associated with the excitatory actions of the amines on the smooth muscle of the blood vessels, uterus, nictitating membrane, dilator pupillae, fundus, stomach, and ureter. For these responses the order of potency was l-adrenaline > dl- adrenaline > noradrenaline > methylnoradrenaline > methyladrenaline > isoproterenol. Beta receptors were associated with the inhibitory actions of the amines on vascular, bronchial, and uterine smooth muscle, as well as with their positive inotropic and chronotropic actions on myocardium.

The order of potency was isoproterenol > ℓ-adrenaline >methyl adrenaline > dℓ-adrenaline > methyl noradrenaline > noradrenaline.

The concept of α- and β-adrenergic receptors was greatly strengthened when specific blocking agents were developed for each receptor (Figure 4). Although α-adrenergic blocking agents had been available for many years, β-adrenergic blocking agents only came into being with the appearance of dichloroisoproterenol (DCI) in 1958 (Powell & Slater). Ahlquist's theory could now be thoroughly tested and it was not only confirmed but extended. Some catecholamines, such as adrenaline, were found to act at both types of receptors; others, such as phenylephrine, were found to act primarily on α-adrenergic receptors. Isoproterenol, on the other hand, was found to have a selective effect on β-adrenergic receptors.

3. Structure-activity relationships

The basic structure of a catecholamine is shown in Figure 2. Much has been learned about adrenergic receptor sites from the use of substituted derivatives. Excellent reviews have been provided (Ariens, 1967; Biel & Lum, 1966; among others) and the main structural requirements for activity at the adrenergic receptor sites are generally considered to be:-

a) Phenolic hydroxyl groups: Both hydroxyl groups are absolutely essential to the production of β-adrenergic receptor stimulation. Elimination of these groups results in complete

lack of activity for β-adrenergic receptors and partial loss of activity for α-adrenergic receptors. The meta-hydroxyl group is more important than the para-hydroxyl group for the action of both types of receptors. Substitution of the OH groups by Cl^-, NO_2, OCH_3, etc. produces compounds which act as β-adrenergic blocking agents.

b) Phenyl ring: The phenyl ring is an absolute requirement for production of β-adrenergic activity, but compounds, such as 2-amino heptane, will still elicit α-adrenergic responses.

c) Beta-carbon atom: The presence of a hydroxyl group on this carbon contributes to direct action on the receptor for both alpha and beta receptors and for their blocking agents as well; therefore, it may be one of the anchor groups by which the catecholamine is bound to the active site. Because this is an asymmetric carbon atom, the steric configuration is important. The ℓ-isomer (or R-isomer, where absolute configuration has been determined) is usually more active than the d-isomer, but not always. They may be equally active. The ℓ/d ratio can vary from 1 to several hundred depending on the species, tissue and effect being measured. The d-isomer can sometimes act as a blocking agent for the opposite type of receptor, protecting it from weak stimulation by the ℓ-isomer. In such cases, the activity of the catecholamine is expressed as the ratio of its sympathomimetic to its sympatholytic activity (mim/lyt) for each type of receptor.

Catecholamines devoid of the OH group on the beta carbon, the dopamine derivatives, have physiological functions of their own. Because this OH group is not necessary to their activity, it is becoming common to speak of dopaminergic receptors.

d) Alpha-carbon atom: Although this carbon atom appears to be necessary for the spacing between the catechol nucleus and the amino group, its hydrogens are unsubstituted in all α- and β-adrenergic compounds. Introduction of alkyl substituents leads to complicated stereochemical relationships. For example, an alpha-methyl substitution results in decreased activity for both α- and β-adrenergic receptors.

e) Amino group: The lack of substitution on the amino group appears to be of primary importance for the intrinsic activity of the α-adrenergic compounds. The unsubstituted compound (noradrenaline) acts mainly on α-adrenergic receptors, whereas the CH_3-substituted compound (adrenaline) acts on both α- and β-adrenergic receptors. The introduction of N-alkyl groups of increasing size results in a decrease in α-adrenergic activity and an increase in β-adrenergic activity. N-aralkyl substituents increase the affinity for β-receptors still more. An example of this type of catecholamine is shown in Figure 2. These catecholamines behave as α-adrenergic blocking agents which implies that they have an affinity for the α-adrenergic receptors but fail to take part in (or produce the proper

conformation for) active site reactions which lead to the physiological response. Substitution with a small, branched, alkyl groups is more effective than substitution with a large, unbranched chain. Thus, the isopropyl and aralkyl derivatives are among the most active of the β-adrenergic compounds.

4. Isoproterenol (mol. wt. 211.24)

1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol (shown in Figure 2) is also known as isoprenaline, isopropylarterenol, N-isopropylnoradrenaline, and many trademarks, such as Aludrine (isoproterenol sulphate), Isuprel, Asmalar, Novodrin, Assiprenol, Respifral, Bellasthman, etc. The patent for its synthesis first appeared in Germany, in 1942. It has medical applications as a bronchodilator and in treatment of heart block (Goth, 1970).

This compound is usually considered to be the prototype of catecholamines which act at β-adrenergic receptors only. The isopropyl substitution on the amino group, the lack of substitution on the alpha-carbon, the hydroxyl group on the beta-carbon and the catechol nucleus, all contribute toward very efficient binding and activating reactions at the β-adrenergic receptor site. Although the ℓ/d ratio for activity of the isomers has been found to be very high for some effects in some tissues (Ariens, 1967) the d-isomer has been shown to be just as active as the ℓ-isomer in producing β-adrenergic responses in the salivary glands (Kirby et al, 1969). The d-isomer was found to

act as a weak α-adrenergic blocking agent (Luduena, 1962) so its presence in dℓ-isoproterenol may actually provide protection against weak agonist activity of ℓ-isoproterenol.

5. Catecholamine interactions with receptors

Catecholamine-receptor interactions, like enzyme-substrate interactions, must include a) "recognition" and binding of the catecholamine to the active site, and b) active participation in the reaction mechanism (or achievement of the proper conformation for the reaction to take place). Several amino acid side chains, with different chemical properties, are involved in known enzyme-substrate interactions. The chemical properties of the catecholamine which might enable it to take part include: i) its ability to participate in oxidation-reduction reactions; ii) its ability to form chelates with ligands supplied by the phenolic oxygens and, in the monoanionic form, to engage in acyl, alkyl and phosphoryl group transfer; and iii) its ability to engage in hydrogen bonding. Many reports deal exclusively with possible reactions at the catecholamine-receptor surface. Because the catechol binding site has a preference for aromatic rings carrying electron-rich substituents, Belleau (1966) suggested that complex formation may be largely dependent on π-bonding. For α-adrenergic activation, a small cationic head (such as ammonium or methyl ammonium) is essential and the bulkier N-substituents (necessary for β-adrenergic activation) would

hinder ion-pair formation. On the other hand, the formation of an active β-receptor complex is independent of the presence or size of the N-substituents. Thus, hydrophobic interactions may be involved in the binding or activation by nonpolar N-substituents at the β-adrenergic receptor. Speculations, such as these, are very interesting but a final conclusion will not be reached until the receptor protein is purified and its reactive groups at the active site determined. Therefore, a great deal of excitement was aroused when it was suggested that the β-adrenergic receptor and adenyl cyclase might be one and the same and that all β-adrenergic effects may be mediated by cyclic AMP (Vane, 1962; Belleau, 1963; Ariens, 1963; Sutherland, 1965).

B. ADENYL CYCLASE AS THE β-ADRENERGIC RECEPTOR

In 1957, while studying the breakdown products of ATP, Cook et al reported the presence of a cyclic anhydrodiadenylic acid. The same year, Sutherland and Rall (1957), while studying phosphorylase activation in liver particles, described the properties of an adenine ribonucleotide produced in the presence of adrenaline or glucagon. In 1958, Sutherland and Rall fractionated and determined the cyclic nature of adenosine 3',5'-monophosphate (cyclic AMP) and it turned out to be the same compound as that described by Cook et al (1957) and Lipkin et al (1959). Although Sutherland and Rall anticipated a cyclizing enzyme (1960), the enzyme catalyzing cyclic AMP formation, by intramolecular condensation of ATP, was not characterized until 1962 by

Sutherland et al. They named it adenyl cyclase and it is often referred to by the chemically correct term, adenylate cyclase. In 1963 it was shown to be a component of the cell membrane (Davoren & Sutherland) or of membranous structures within the cell, such as sarcoplasmic reticulum (Rabinowitz et al, 1965). The particulate nature of adenyl cyclase, together with its lability, have combined to make its purification very difficult. Therefore, we are not much closer to discovering specific receptor reactions.

Among the hormones which are known to stimulate adenyl cyclase in various tissues, the catecholamines were found to figure prominently, for example, in cardiac muscle (Murad et al, 1962); in liver (Makman & Sutherland, 1964); in skeletal muscle (Posner et al, 1965); in brain (Kakiuchi & Rall, 1965); in adipose tissue (Butcher et al, 1965); in lung and spleen (Klainer et al, 1962), etc. The order of potency of the catecholamines in cerebellar preparations of the brain (Klainer et al, 1962) and heart and liver preparations (Murad et al, 1962) suggested the participation of β-adrenergic receptors.

The hormonal responses elicited by catecholamines and found to involve cyclic AMP include phosphorylase activation in liver (Haugaard & Hess, 1965), skeletal muscle (Krebs et al, 1966), and heart (Drummond & Duncan, 1966); a positive inotropic effect on the heart (Robison et al, 1965); lipolysis

(Butcher, 1966); and the release of α-amylase in saliva from rat parotid gland slices (Bdolah & Schramm, 1965).

The real test for the hypothesis involves the effects of β-adrenergic blocking agents (Figure 4) on adenyl cyclase activity. Here the evidence was far from complete; however, a few studies appeared to support the theory. In 1962, Murad et al, using particulate fractions of dog heart and liver, reported that DCI prevented the stimulating effects of catecholamines on heart adenyl cyclase almost completely. A few years later, Robison et al, (1965) showed that another β-adrenergic blocking agent, pronethalol blocked the increase in cyclic AMP concentration and the positive inotropic effect produced by adrenaline on the isolated, perfused rat heart. A third study involving β-adrenergic blocking agents was one by Davoren and Sutherland (1963a) in which DCI was found to inhibit the formation of isoproterenol-induced cyclic AMP almost completely in pigeon erythrocytes.

The literature concerning adrenergic responses was becoming particularly confusing and contained many contradictory statements. The main problems were reviewed by Robison et al, (1967) so will be mentioned only briefly here. In the first place, variation in response occurred from species to species. The stimulation of liver glycogenolysis, which in the dog appeared to be mediated by β-receptors, in the rat

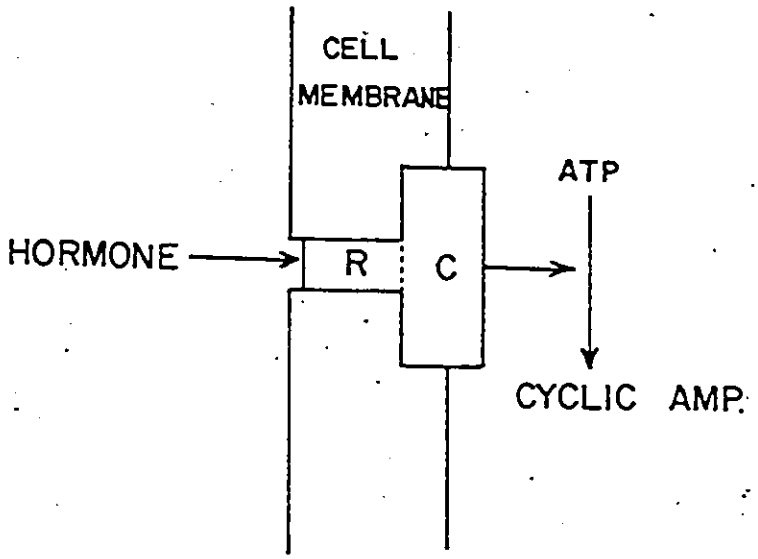


FIGURE 3. HYPOTHETICAL REPRESENTATION OF THE ADENYL CYCLASE MOLECULE. From Robison et al, Ann. N.Y.Acad.Sci.139:795 (1967)

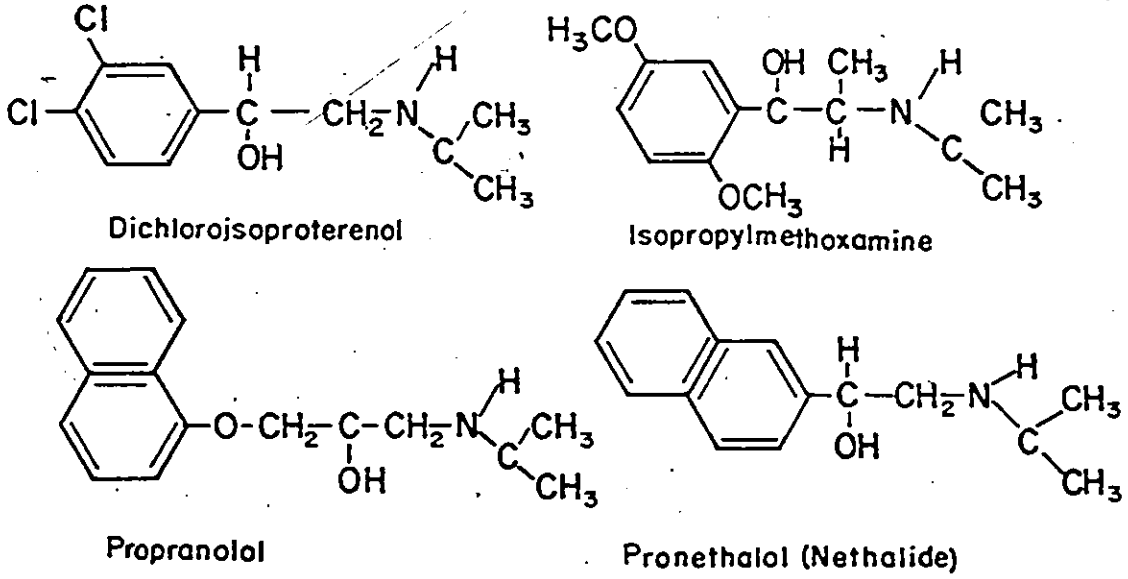


FIGURE 4. BETA-ADRENERGIC BLOCKING AGENTS

was found to be an α-adrenergic response. There were variations in properties from tissue to tissue and even within a given type of tissue. Responses defined as beta in heart and uterus responded differently to noradrenaline. The blocking agent, isopropylmethoxamine, was found to block β-receptors in uterus but had only a slight effect on heart receptors.

In addition, there were variations in over-all response. In some cases, as in intestinal smooth muscle, stimulation of either type of receptor was found to cause relaxation. Also, changes in physiological or pathological conditions were found to produce varied responses. For example, in rabbit, alpha receptors predominated in the virgin uterus, but in the progesterone-proliferated uterus, beta receptors appeared to predominate. The opposite was true for cat uterus.

To add to the confusion, when metabolic effects were studied in different tissues and species, differing orders of potency and different susceptibilities to blockade were observed (Hagen & Hagen, 1964). Some authors suggested that the metabolic effects had no relation to the mechanical effects upon which the classification of alpha and beta adrenergic receptors was originally based (Ahlquist, 1948).

In 1967, some evidence began to appear to support the view that the effects of α-receptor activation might be related

to a decrease in the intracellular level of cyclic AMP, either by inhibition of adenyl cyclase or by activation of a phosphodiesterase, the enzyme which destroys cyclic AMP. This was suggested by Turtle et al, (1967) on the basis of experiments showing the effects of theophylline (a methyl-xanthine inhibitor of phosphodiesterase) and adrenergic blocking agents on insulin release. The work of Porte (1967) on α-adrenergic receptor inhibition of insulin release by adrenaline was also significant. The implications of these findings were great because some of the observations which appeared to contradict the accepted definitions of receptor types could now be explained.

In 1967, Robison et al decided that the variations in species and tissue responses were problems common to most enzymes and that enough circumstantial evidence had accumulated to warrant consideration of the hypothesis that both alpha and beta receptors operate through the adenyl cyclase system.

In constructing a model to explain observed variations in response, Robison et al, (1967) favoured the view that the receptor is an integral part of the adenyl cyclase system. Receptors were thus coming to be regarded as analogous to the allosteric sites known to be part of certain enzymes. In this model (Figure 3) adenyl cyclase is pictured as existing in the

cell membrane, as suggested by the data of Davoren and Sutherland (1963b). The molecule was depicted as being composed of at least two distinct subunits, a regulatory subunit (R), facing the extracellular fluid, and a catalytic subunit (C), the active centre of which was in contact with the interior of the cell. The actual receptor was considered to be part of the regulatory subunit which was the variable component of the system, differing to some extent from tissue to tissue so that differing potencies of the catecholamines could be related to differences in affinity (and to account for the fact that other hormones also stimulate adenyl cyclase). The tentative conclusion was that all metabolic effects of catecholamines could be accounted for by changes in the concentration of cyclic AMP in the affected cells although Robison et al (1970) emphasized that the mechanism by which an alteration in one type of subunit leads to a change in the other was poorly understood.

C. CYCLIC AMP: THE SECOND MESSENGER

Attention is now focused on the mechanisms whereby cyclic AMP levels are translated into metabolic and physiological events. As mentioned earlier, even though the receptor protein has been identified there can still be numerous reactions occurring sequentially before the characteristic response of the hormone is observed. Cyclic AMP became known as "the second messenger" in the mediation of a variety of hormonal effects (Robison

et al, 1968). Despite the widespread distribution of functions attributed to cyclic AMP, knowledge of its mechanism of action is only gradually unfolding. The sequence of biochemical steps occurring at the molecular level was first resolved in the area of glycogen metabolism.

Activation of phosphorylase b kinase

The activation of phosphorylase was discovered to be mediated by cyclic AMP by Rall et al (1957) while they were studying the stimulation of glycogenolysis by adrenaline and glucagon. The sequence of biochemical steps, as shown in Figure 5, was gradually worked out over an 11-year period. Phosphorylase was known to exist in an inactive state (phosphorylase b) which could be phosphorylated by phosphorylase kinase, acting on ATP to produce the active phosphorylase a. Phosphorylase b kinase was also found to exist in two interconvertible forms, designated as non-activated and activated phosphorylase b (Krebs et al, 1959) and cyclic AMP was found to promote the formation of activated phosphorylase b kinase (Krebs et al, 1964). Conversion of nonactivated phosphorylase b kinase to the activated form required ATP and was catalyzed by phosphorylation involving another enzyme (Delange et al, 1968). Therefore, although cyclic AMP appeared to be necessary for activation of both phosphorylase b kinase and phosphorylase b, the actual receptor protein for cyclic AMP was a protein kinase, phosphorylase b kinase kinase.

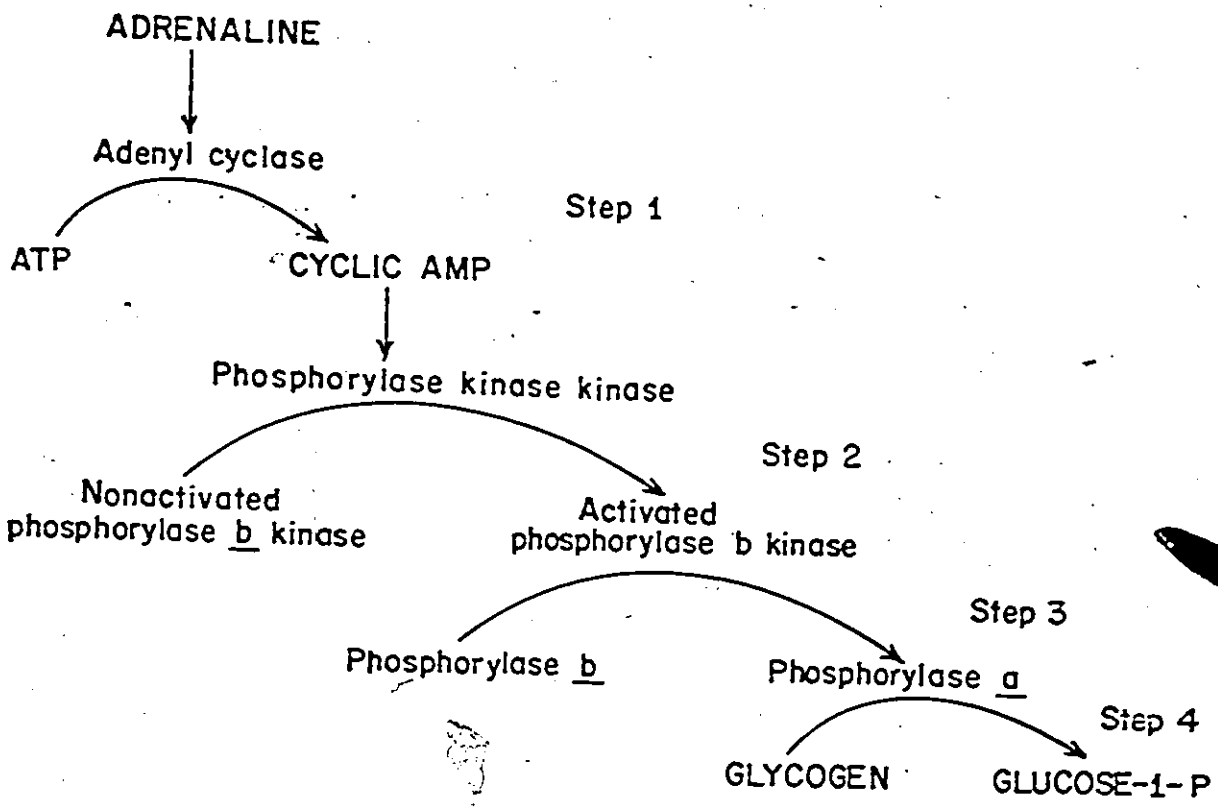


Figure 5: SEQUENCE OF STEPS IN GLYCOGENOLYSIS

1: ADRENALINE (OR NORADRENALINE AT NERVE ENDINGS) STIMULATES ADENYL CYCLASE IN THE MEMBRANE TO FORM CYCLIC AMP.

2. CYCLIC AMP ACTIVATES A PROTEIN KINASE WHICH CATALYZES THE PHOSPHORYLATION OF PHOSPHORYLASE KINASE.

3. PHOSPHORYLASE KINASE CATALYZES THE CONVERSION OF PHOSPHORYLASE b TO PHOSPHORYLASE a.

4. PHOSPHORYLASE a CATALYZES THE FORMATION OF GLUCOSE-1-PO_4.

D. CYCLIC AMP-DEPENDENT PROTEIN KINASE

Cyclic AMP-dependent phosphorylase b kinase kinase was first discovered in crude extracts of rabbit skeletal muscle from which phosphorylase b kinase had been removed by acid precipitation. It was partially purified and found to phosphorylate casein and protamine as well as phosphorylase b kinase, so was given the general name of cyclic AMP-dependent protein kinase by Walsh et al, in 1968. The same year, Langan (1968) reported the hepatic histone kinase which he was studying, to be stimulated by cyclic AMP also. It has since been reported to be present in many tissues, including adipose tissue (Corbin & Krebs, 1969); brain (Miyamoto et al, 1969); bladder (Jard & Bastide, 1970); testis (Jergil & Dixon, 1970); mammary gland (Waddy & Mackinlay, 1971); pancreas, kidney, lung, thyroid, ovary, stomach, duodenum, uterus, brown adipose tissue (Kuo & Greengard, 1969a); and parotid gland (Horwood et al, 1971). It is also found in bacteria (Kuo & Greengard (1969b).

1. Functions

Protein kinase catalyzes the transfer of phosphoryl groups from ATP to certain proteins. The phosphoryl groups are bound in ester linkages to serine and generally to threonine as well. The various groups of protein kinases may be distinguished by identifying the protein which is preferentially phosphorylated.

There is now evidence that, in addition to stimulating the phosphorylation of phosphorylase b kinase, cyclic AMP also

stimulates the phosphorylation of glycogen synthetase (Bishop & Larner, 1969); a hormone-sensitive lipase in adipose tissue (Corbin et al, 1970; Huttunen et al, 1970); histone in liver (Langan, 1969); pyruvate dehydrogenase in heart (Weiland & Siess, 1970); protamine in trout testis (Jergil & Dixon, 1970); phosphorylase phosphatase in muscle (Chelala & Torres, 1969); and the 40S and 60S ribosomal subunits in liver (Eil & Wool, 1971).

2. Properties

a) Substrate specificity: The protein kinases which phosphorylate the enzymes listed above will transfer phosphoryl groups from ATP to a seryl or threonyl residue of basic proteins, such as histone and protamine, and will phosphorylate casein, though not as extensively as the basic proteins. Most of the enzymes listed in Table I, (1) to (13) are of this type and are cyclic AMP-dependent. The second main type of protein kinase phosphorylates the seryl and threonyl groups of acidic proteins, such as casein and phosvitin, and is not dependent on cyclic AMP (Walinder, 1972). This type of protein kinase was studied for some time before the discovery of the cyclic AMP-dependent type (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960). It is associated with nuclear (non-histone chromatin) phosphoproteins (Takeda et al, 1971; Ahmed, 1971), and can be distinguished from nuclear histone kinase because it is insensitive to sulfhydryl agents and is inactive with histone as substrate (Langan & Smith, 1967). Multiple forms of this enzyme have been reported (Ruddon and Anderson, 1972; Baggio & Moret, 1971).

TABLE I

SOME CONDITIONS FOR PROTEIN KINASE ACTIVATION

SHOWN IN STUDIES FROM THE LITERATURE

Authors	Tissue and fraction where found	pH optimum	Preferred substrates	cAMP activated	Mg^{2+} required	Period linear
					mM	min
(1) Walsh et al, (1968)	Rabbit skeletal muscle, soluble fraction	6.0	Phosphorylase kinase	Yes	3.6	1
			Casein	Yes		
			protamine	Yes		
(2) Reimann et al, (1971)	Rabbit skeletal muscle, 10,000 g supernatant	6.0	Casein	Yes	10	20
			Histone	Yes		
			Glycogen synthetase	Yes		
(3) Langan (1968)	Liver nuclei	7.5	Histone	Yes	5	25
(4) Kuo et al (1970)	15 bovine tissues	6.5	Histone	Yes	10-20	8
(5) Jard & Bastide (1970)	Frog bladder epithelial cells	7-8.0	Histone	Yes	10	10
(6) Tao et al, (1970)	Rabbit reticulocytes, 100,000 g supernatant	7.5	Histone	Yes	20	
			Casein	No		
(7) Waddy & Mackinlay (1971)	mammary gland, 16,000 g supernatnat	8.0	Histone	Yes	2-4	20
(8) Corbin et al, (1972) (1973)	Rat epididymal fat pads, 8,000g infranatant, then 27,000 g supernatant	6.5	Casein	Yes	6	
			Histone	Yes		

TABLE I (Continued)

Authors	Tissue and fraction where found	pH optimum	Preferred substrates	cAMP activated	Mg^{2+} required	Period linear
					mM	min
(9) Maeno et al, (1971)	Rat cerebral fractions	6.5	Histone	Yes	10	
(10) Miyamoto et (1969)	Bovine brain, 27,000 g supernatant	6.5	Histone	Yes	10-40	10
			bovine albumin	Yes		
			casein	Yes		
			protamine	Yes		
(11) Johnson et al,(1971)	Rat brain synaptic plasma membrane & microsomal fr.	6.0	intrinsic	Yes	10	10
			Histone	Yes		
(12) Erlichman et al,(1971)	Bovine heart, supernatant	7.0	protamine	Yes	10	10
(13) Soderling et al, (1970)	Rabbit skeletal muscle soluble fraction	6.8	glycogen synthetase	Yes	1.25	1
			phosphorylase kinase	"		
		6.7	Histone	Yes	10	
		6.0	Casein	Yes		
(14) Desjardins et al,(1972)	Rat liver nuclei	6.0	Phosvitin	No	8-15	
			Histone	No		
(15) Weller & Rodnight(1971)	Ox brain synaptosomal and microsomal fractions	7.3	intrinsic	Yes	0.15	1
			phosvitin	No		
(16) Walinder(1972)	Calf brain	7.5	phosvitin	No	5	
			Casein	No		

b) pH optimum: As shown in Table I, there is great variation in the pH optima reported for protein kinases from different tissues. It appears to vary with the type of substrate used. In general, pH optimum for histone phosphorylation is between 7.0 and 8.0 (5, 6). The pH optimum for casein phosphorylation is close to 6.0 (2) and is not cyclic AMP-dependent at pH 7.5 (6). On the other hand, cyclic AMP-stimulated phosphorylation of histone occurs over the whole spectra of pH values (Table I).

c) Cyclic AMP-dependence: When activity was first measured as a function of cyclic AMP concentration, activity of the enzyme was found to reach a maximum at 5 μM in many tissues (Kuo et al, 1970). The apparent K_M ranged from 30 to 160 nM. The extent of stimulation by cyclic AMP was shown to vary depending on the tissue from which the enzyme was isolated, the particular substrate used and the length of storage. Protein kinase from skeletal muscle (Walsh et al, 1968) had a much higher degree of dependency on cyclic AMP than that of heart (Brostrom et al, 1970) or of adipose tissue (Corbin & Krebs, 1969). Protein kinase of heart muscle was found to be completely independent of cyclic AMP upon storage of 4 to 6 weeks. Because of findings such as these, the mechanism of activation was determined and it is now known that a K_M value cannot be assigned unless the ratio of free to bound protein kinase is known.

d) Apparent K_M for ATP: Double reciprocal plots of ATP concentration against rate of ^{32}P incorporation into protein showed that the K_M for ATP in many tissues was close to 1.1×10^{-5} both

in the presence and absence of cyclic AMP but the V_{max} increases many times with added cyclic AMP. In some cases a biphasic reciprocal plot was obtained in the absence of cyclic AMP and this was interpreted as being due to a mixture of two types of protein kinase differing in their apparent Michaelis constants for ATP. Reimann et al,(1971), using DEAE-cellulose to separate peaks of enzyme activity, showed that two enzymes, each with a K_M close to 1.1×10^{-5} for ATP, were present and a third, which was not cyclic AMP-dependent had a K_M for ATP greater than 10^{-4}.

e) Mg^{2+} requirement: An absolute requirement for Mg^{2+} is reported by most investigators, usually around 10 mM, as shown in Table I. Mg^{2+} has been found to decrease the apparent K_M for ATP in skeletal muscle without changing the V_{max} (Reimann et al,1971). They suggested that Mg^{2+} may bind to the enzyme at more than one site and serve in a regulatory capacity. In the study in which only 0.15 mM $MgCl_2$ was used, the assay required up to 3 mM ATP (Weller & Rodnight, 1973).

f) Effect of NaF: In all studies listed in Table I, except (3), (8), and (14), NaF is included in the protein kinase assay. It has been shown to inhibit phosphatase activity and if it is not included, the reaction is linear for only 1 min (Corbin et al, 1973), especially in particulate fractions containing membrane-bound ATPase. Sodium fluoride (approx. 50mM) was found to increase the cyclic AMP-activation of protein kinase (Corbin et al, 1973) whereas the same concentration was found to be inhibitory in ox brain synaptosomal fraction (Weller & Rodnight, 1973). The

latter also show that phosphatase activity is highest at pH 7.3 (Weller & Rodnight, 1971) and decreases by 60% at pH 6.5.

g) Phosphodiesterase inhibitors: All investigators listed in Table I, except (3),(8), and (14), include a phosphodiesterase inhibitor in the assay, usually 2mM theophylline. Weller and Rodnight (1973) report that 5mM theophylline inhibits their protein kinase activity whereas caffeine is shown to have no effect at all on adrenaline-induced activation of protein kinase in rat adipose tissue (Soderling et al, 1973).

h) Effects of salt: The effects of physiological concentrations of NaCl and KCl (150mM) have been tested and NaCl (100mM) and KCl (100mM) were both found to inhibit the cyclic AMP-stimulated intrinsic protein kinase activity in membrane fractions from ox brain (Weller & Rodnight, 1973). On the other hand, NaCl (0.5 M) was found to have a stimulatory effect on adipose tissue enzyme of crude fractions (Corbin et al, 1973). This was discovered to be due to a slow dissociation of the inactive protein kinase complex and release of the active form of the enzyme.

i) Effects of EGTA and EDTA: In all studies shown in Table I, except 1,5,8,12 and 13, EGTA (usually 0.3mM) is added to the assay, or EDTA is used in the homogenizing medium. Because divalent cations, such as Ca^{2+} have been shown to inhibit protein kinase activity (Weller & Rodnight, 1971), there is good reason to remove all endogenous Ca^{2+}. In this way the amount of divalent cation in the assay can be controlled.

3. Mechanism of activation of protein kinase

The action of cyclic AMP in the stimulation of protein kinase has been studied by investigating the binding of the nucleotide to the enzyme and measuring the kinetic parameters. Because cyclic AMP increased the V_{max} but had no effect on the K_M, and because oxidation or dilution of the enzyme resulted in a structural change similar to that induced by cyclic AMP in binding to the enzyme, it was proposed by several investigators, independently, that protein kinase consists of a regulatory (R) and a catalytic (C) subunit, which dissociate in the presence of cyclic AMP (Tao et al.,1970; Brostrom et al.,1970; Gill & Garren, 1970; Kumon et al.,1970; and later by other groups of workers). The cyclic AMP is believed to bind to the holoenzyme (R.C) in such a way as to cause dissociation of the catalytic subunit (C), which is the active form of protein kinase and no longer dependent on cyclic AMP:

$$\underset{\substack{\text{(holoenzyme)}\\\text{(inactive form)}}}{R \cdot C} + cAMP \rightleftharpoons R \cdot cAMP + \underset{\substack{\text{(active form of}\\\text{protein kinase)}}}{C}$$

Brostrom et al.,(1971) presented evidence to show that (C) causes displacement of cyclic AMP bound to (R); in other words, an excess of (C) will cause the reaction to shift to the left. Similarly, an increase in the cyclic AMP level, will shift the equilibrium to the right, resulting in an increase of the active,dissociated protein kinase. If the level of cyclic AMP falls, then more of the inactive complex results. In effect, the (R) subunit serves as an intracellular reservoir of cyclic AMP. The active form of

protein kinase was found to be inhibited by the addition of the regulatory subunit (Yamamura et al, 1971; Tao et al, 1970; Gill & Garren, 1971; Erlichman et al, 1971).

Soderling et al, (1973) incubated fat pads with adrenaline and demonstrated that the ratio of free protein kinase to bound protein kinase increased. When adrenaline was replaced by insulin, the ratio was found to decrease. This, in effect, proves the hypothesis of Robison et al, (1967) that hormonal responses are mediated at adrenergic receptors by an increase or a decrease in the level of cyclic AMP.

It has also been shown that basic proteins, such as histone (Miyamoto et al, 1971) and protamine (Tao, 1972) have the ability to stimulate protein kinase by dissociating the C.R subunit in the same manner as cyclic AMP. There are two schools of thought regarding the role of histones in vivo. One involves a cyclic AMP-dependent protein kinase and the other does not. Langan (1969) demonstrated that histones are phosphorylated in vitro and in vivo by a cyclic AMP-dependent protein kinase. Histones were also reported to cause a large increase in the rate and extent of phosphorylation of nuclear acidic (non-histone chromatin) proteins (Kaplowitz et al, 1971). The histone, itself, was not believed to be phosphorylated, but rather, to act in a way such as to increase the number of phosphorylation sites available in the phosphoprotein. Rates of phosphorylation of acidic nuclear proteins were shown to be increased in cells undergoing gene

activation and preceding an elevation of RNA and protein synthesis (Teng et al, 1971). Some hormones, such as testosterone, were found to have a direct effect on nuclear phosphoproteins (Ahmed & Ishida, 1971). Within 30 min [γ-^{32}P]-ATP was incorporated into phosphoproteins of rat ventral prostate nuclei.

Now the pendulum of thought appears to be swinging back to involve cyclic AMP in nuclear functions once more. Dokas and Kleinsmith (1971) report that cyclic AMP increases the capacity for RNA synthesis in rat liver nuclei and that induction of enzyme synthesis, mediated by cyclic AMP, may be controlled, at least in part, at the level of gene transcription. In addition, Weller and Rodnight (1973) state that although the protein kinase found in their synaptic membrane fragments is not activated by cyclic AMP during phosphorylation of added acidic proteins, such as phosvitin; the intrinsic activity, in phosphorylating the natural substrate, is cyclic AMP-dependent. This brings us back to one common denominator, a cyclic AMP-dependent protein kinase. This protein kinase may, in the final analysis, activate acidic or basic proteins equally well in vivo, even though it does not appear to do so in vitro (Table I, 14-16).

PART II: THE SALIVARY GLANDS

A. GENERAL DESCRIPTION

1. Anatomy

In many animals, including the rat, the salivary glands consist of three paired structures with ducts to carry saliva to the oral cavity; thus, salivary glands are glands of external secretion or exocrine glands. The main ones are called the parotid, submandibular (formerly known as the submaxillary) and the sublingual. In addition there are numerous small salivary glands scattered over the oral mucosa. In the rat, the submandibular glands lie longitudinally near the midline of the neck and extend from the hyoid bone to the manubrium sternii. The major sublingual lies in close apposition to the submandibular on its antero-lateral surface and the two are contained within a single capsule. The parotid is lighter in colour than the submandibular and tri-lobed, with one section extending medially to the anterior surface of the submandibular, another extending posteriorly, and a third extending superiorly behind the ear.

The salivary glands are typical of compound tubular glands in which the glandular cells are arranged in a single layer around a central cavity which receives the secretion

from surrounding cells. The unit is called an acinus or an alveolus. Small ducts from adjacent acini join together to form larger ducts.

Histologically, the parotid gland consists almost entirely of serous cells which are small and granular with well-stained nuclei. Saliva from this gland is clear and watery and is much less viscous, but richer in α-amylase and other enzymes, than that from other salivary glands. The sublingual gland secretes a thick, sticky, opalescent material, rich in lubricating glycoproteins which are synthesized in mucous cells. Mucous cells are larger than serous cells but are clear and transparent and are the main type of cell in the sublingual gland. The submandibular gland contains both serous and mucous acini in roughly equal proportions.

2. Innervation

Salivary glands are unique in that their physiological functions are controlled through the autonomic nervous system. In 1851 Karl Ludwig discovered that saliva flows from the submandibular gland both when the chorda-lingual nerve of the parasympathetic branch and when the cervical sympathetic trunk are stimulated electrically so it has been known for some time that salivary glands are supplied by both branches of the autonomic nervous system. However, it has now been established that adrenergic innervation reaches the acinar cells in both rat parotid and submandibular glands but not

those of the sublingual gland (Norberg & Olson, 1965); furthermore, there are α-adrenergic and β-adrenergic receptors in both rat parotid and submandibular glands (Emmelin et al.,1965; Batzri et al.,1971) and release of K^+ by α-adrenergic stimulation can be prevented by phentolamine.

3. Salivary enzymes

The composition of the saliva depends on the branch of the autonomic nervous system which is stimulated. In general, the parasympathetic nerves control the flow rate and the sympathetic nerves control the secretion of digestive enzymes (Pohto, 1968). Salivary glands synthesize at least five different digestive enzymes for excretion, including α-amylase, protease, lysozyme, DNase and RNase (Junqueira, 1967). The salivary content of these enzymes was studied in more than 45 species. Rodents were found to have the highest amounts of all salivary enzymes and the genus Rattus was the most advanced in this respect. Primates (including Homo sapiens) have only 25% of the salivary enzyme activity of rodents.

There is also a difference in the proportion of each enzyme in the same gland of different species. For example, mouse submandibular gland contains α-amylase but rat submandibular is not considered to be an α-amylase producing gland (Schneyer & Schneyer, 1964). Rats of the suborder, Myomorpha have very high levels of α-amylase and DNase in the parotid and protease in the submandibular (Junqueira, 1967).

4. Model for the study of β-adrenergic receptors

Although the natural functions are important to our well-being, they are not the reason for which we study salivary glands. Because of its high content of α-amylase, its low content of mucous and its distinctive β-adrenergic responses, the rat parotid provides a unique model for studying the sequence of biochemical reactions leading to excretion, synthesis and storage of excretable proteins. In addition, the processes of hypertrophy and hyperplasia can be studied through stimulation of β-adrenergic receptors. The stimulus provided by a single injection of chemically pure isoproterenol produces a response involving a large fraction of the cell population and a very attractive model is provided for studying DNA-dependent RNA synthesis. Some β-adrenergic effects can be studied in rat submandibular gland, as well, because it has sympathetic innervation, although it does not synthesize α-amylase.

B. EFFECTS OF ISOPROTERENOL

To sort out the effects due entirely to β-adrenergic receptor stimulation, we use isoproterenol because it has very little, if any, action on α-adrenergic receptors, as discussed in Part I. It is useful, also, in sorting out adrenergic effects from cholinergic effects. Much confusion had arisen because cholinergic agents, such as pilocarpine, were

thought to act at ganglia to produce β-adrenergic effects (Schneyer & Hall, 1966) in parotid and submandibular glands which contain both adrenergic and cholinergic nerve endings.

1. Hypertrophy

In 1960, Wells reported that considerable hypertrophy of the salivary glands (Sialadenosis) resulted from chronic stimulation of the sympathetic nerves. He concluded that the sympathetic nervous system is involved in the maintenance of normal gland size and function. The following year, two reports appeared, each dealing with excessive stimulation of salivary gland growth due to chronic treatment with isoproterenol (Selyé et al.,1961; Brown-Grant, 1961). Brown-Grant (1961) and Schneyer (1962) reported that the parotid gland shows the greatest enlargement (550% by the 8th day) and that the sublingual gland is not affected. The hypertrophy was found to recede if isoproterenol is withdrawn for 10 days (Wells, 1962).

Brown-Grant (1961) stated that the most striking feature, in both parotid and submandibular glands, is a great increase in the size of the acinar cells. Histological studies by Seifert (1967) showed that the mean diameter of the acinus increases from 25μ (controls) to maximal values of 50μ and that the nuclei and nucleoli increase in size as well

Although Brown-Grant, Schneyer and Wells attributed the enlargement of the glands principally to cellular hypertrophy, Selyé et al, (1961) had observed, by histological examination, mitotic proliferation as well; thus a certain amount of controversy developed. Special consideration was given to weight and structure of salivary glands by many investigators, such as Schneyer & Shackleford (1963), Pohto & Paasonen (1964), Argonz (1962), etc.

2. Hyperplasia

The fact that isoproterenol could induce cell proliferation was indeed confirmed (Chan, 1964; Barka, 1965a). Histological studies by Seifert (1967) showed that the mitotic index of the parotid gland, which is normally lower than 0.5% in controls, rose to 1.5% on day 4, then declined, but began to increase a second time, reaching a peak of 3.2%,or higher,by day 11 or 12. After establishment of mitotic cell division occurring in salivary glands, a flurry of reports appeared regarding efforts to establish the intramolecular series of biochemical steps leading to DNA synthesis, RNA synthesis and related protein synthesis. Competition was between two main groups, headed by Barka and Baserga.

a) DNA synthesis: A single injection of isoproterenol was found to produce a marked stimulation of DNA synthesis in salivary glands of rats (Barka, 1965b) and mice (Baserga, 1966).

DNA synthesis was measured by the rate of incorporation of ^{3}H-thymidine into DNA and by autoradiographic techniques (Seifert, 1967; Radley, 1968). The increase in DNA synthesis begins about 20 hours after administration of isoproterenol, reaches a peak at about 28 hours, and is followed by a net increase in the amount of DNA per gland and by a wave of mitosis. The peak of DNA synthesis is closely paralleled by changes in the levels of activity of the enzyme deoxythymidine kinase (Whitlock et al, 1968) and deoxythymidylate kinase (Pegoraro & Baserga, 1970). The peak of deoxythymidylate synthetase was found to occur 3 hours earlier (Pegoraro & Baserga, 1970). DNA polymerase activity was also investigated because it reflects the number of cells in the DNA synthetic phase (Whitlock et al, 1968; Barka, 1965b).

b) RNA synthesis: To prove that DNA-dependent RNA synthesis was occurring, the incorporation of ^{3}H-uridine and ^{3}H-orotic acid into total and nuclear RNA fractions was measured by Barka (1966, 1968), and found to peak during the prereplicative stage. This was refuted by Malamud and Baserga (1969) who showed that their increases resulted from corresponding changes in the specific activity of the immediate precursor, UTP. However, another group (Mayfield et al, 1969) also found an increase in the incorporation of ^{3}H-uridine into RNA at 2 hours after isoproterenol, along with increases in the activity

of enzymes involved in pyrimidine biosynthesis. The activity of the enzyme, uridylate kinase was found to decrease between 2 and 8 hours after isoproterenol (Malamud & Baserga, 1968). The possibility of two separate periods of induced RNA synthesis was suggested by Simpson and Baserga (1971) when they discovered that 5-azacytidine inhibits RNA synthesis between 0-2 and 12-18 hours after isoproterenol.

3. Induction of excretion

Cholinergic effects on salivary secretion were always considered more important than adrenergic effects (Burgen & Emmelin, 1961) until 1966 when Byrt showed that a single injection of isoproterenol can cause excretion of 98% of the α-amylase present in the rat parotid. The adrenergic and cholinergic depletory responses were clearly defined by Pohto (1968). The cholinergic agent, pilocarpine, causes a very large flow of saliva containing a small amount of α-amylase and isoproterenol causes a small flow containing a large amount of α-amylase. Pohto (1967) also showed that β-adrenergic receptor control is involved because propranolol partially inhibited excretion of α-amylase, in the manner of a competitive inhibitor. The catecholamine-controlled excretion of α-amylase was the subject for a doctoral thesis in this laboratory (Gaunce, 1971). It was shown that the rate

of excretion of α-amylase by the rat parotid gland reaches a maximum within 10 min after isoproterenol stimulation.

Following isoproterenol injection, dynamic changes in the ultrastructure of the acinar cells of rat parotid are observed during the secretory cycle (Amsterdam et al, 1969). Depletion of the zymogen granules during the first hour is observed to occur by fusion of the granule membrane with the lumen membrane and discharge of the contents into the lumen, which enlarges. No zymogen granules remain after 1 hour. At 2 hours, small, smooth vesicles begin to form in the apical part of the cell and by 6 hours, large condensing granules surround the Golgi membranes and zymogen granules are beginning to form. By 11 hours many zymogen granules have accumulated and by 20 hours, the cell is densely packed and the lumen is very narrow again.

4. Effects on protein synthesis

Protein synthesis is measured by changes in the incorporation of ^{14}C-amino acids into tissue proteins. To separate isoproterenol-induced changes related to hypertrophy and hyperplasia from those related to the secretory processes, antibiotic inhibitors are valuable tools.

a) Antibiotic inhibitors: Protein synthesis controlled at the transcriptional level can be inhibited by actinomycin D. It acts by binding to DNA, by hydrogen bonding to guanine residues, so that formation of messenger RNA cannot proceed (Brockman & Anderson, 1963). Protein synthesis which occurs on preformed RNA,

at the translational level can be inhibited by puromycin. Puromycin interrupts peptide-chain elongation by virtue of its capacity to replace an entering aminoacyl-transfer-RNA with formation of a peptidyl-puromycin derivative. The peptidyl-puromycin so formed cannot be lengthened by a similar displacement reaction with the next aminoacyl-transfer RNA because its amide linkage is substituted and cannot be attached (Nathans, 1964). Another protein synthesis inhibitor, cycloheximide (Actidione) inhibits protein synthesis in the 80S ribosomes of eucaryotic cells (Fiale & Davis, 1965).

b) Secretory proteins: The biosynthesis of α-amylase in rat parotid after injection of ^{14}C-amino acids, was studied by Gromet-Elhanan and Winnick (1963) with feeding (30 min) and pilocarpine injection as stimulators of salivary secretion. It was shown that α-amylase is synthesized in the microsomes, rapidly released into the cytosol, and transferred to the zymogen granules where it is concentrated and stored. Schramm and Bdolah (1964) showed, by pulse-chase experiments that rat parotid slices in vitro maintain very active protein synthesis. Stimulation of parotid slices using adrenaline (Grand & Gross, 1969) was shown to stimulate secretion of α-amylase and protein synthesis independently. By using actinomycin D they also showed that the biosynthesis of α-amylase

proceeds in the absence of RNA synthesis (Grand & Gross, 1970) so they suggested that α-amylase and protein synthesis are controlled at the translational level. Puromycin (200 mg/kg) was found to inhibit incorporation of ^{14}C-amino acids into α-amylase biosynthesis following isoproterenol treatment (Gaunce, 1971).

c) Hypertrophy and hyperplasia: The stimulation of DNA synthesis by isoproterenol was found to be inhibited by both actinomycin D (Baserga & Heffler, 1967; Barka, 1965b) and puromycin (Baserga, 1966) especially when administered prior to, or shortly after, isoproterenol. The incorporation of ^{3}H-uridine and ^{3}H-orotic acid was also found to be sensitive to actinomycin D (Barka, 1966). Cycloheximide, which is particularly effective for the first few hours, was most effective in inhibiting amino acid incorporation into the free ribosome fraction of mouse salivary glands within 1 hour after isoproterenol (Sasaki et al, 1969) so it was suggested that a protein (or proteins) is synthesized at that time which is relevant to the onset of DNA synthesis several hours later. Actinomycin D was found to inhibit an increase in acidic nuclear proteins at 8 and 12 hours but not the increase in the rate of synthesis of nuclear proteins which occurs at 2 hours after isoproterenol (Stein & Baserga, 1970). This temporal difference in the synthesis of histones and acidic (nonhistone) nuclear proteins lead to the suggestion that

functional differences in the two types of nuclear proteins may exist. Acidic nuclear proteins might be involved in the control of cell proliferation in mammalian cells. In contrast, Ekfors and Barka (1971) reported that the synthesis of a group of sedimentable proteins was inhibited by actinomycin D during the first 3 hours after isoproterenol, when the overall synthesis of proteins is depressed in rat submandibular gland. The activities of several enzymes involved in DNA synthesis were also found to be inhibited by actinomycin D (Barka, 1965b; Whitlock et al, 1968; Pegoraro & Baserga, 1970). The possibility exists that enzymes involved in DNA synthesis might be controlled at the transcriptional level while those involved in excretion might be controlled at the translational level.

5. Other effects of isoproterenol

An increase in glycogen storage in mouse salivary glands was claimed by Malamud (1967) and was found to precede DNA synthesis. Although the secretory process is critically dependent on energy, it has been shown that parotid gland ATP is produced by oxidative phosphorylation at a rate fifty times faster than by glycolysis (Feinstein & Schramm, 1970). Salivary gland enzyme excretion is accelerated by inosine and adenine. According to Batzri and Selinger (1973) the purines aid in maintaining the ATP supply. An increased synthesis of glycolipids is reported by Galanti and Baserga (1971). Isoproterenol was reported to cause

the secretion of sialoproteins from submandibular and sublingual glands of rats (Byrt & Glanvill, 1967) but this sounds impossible as sublingual glands have no sympathetic innervation.

6. Structure-activity relationships

There appears to be little doubt that isoproterenol acts at β-adrenergic receptors to produce effects on salivary gland growth, cell division and induction of excretion, however, from the actions of blocking agents, there is some evidence that the receptor sites may not be identical for all three responses. Isoproterenol-induced hypertrophy can be prevented by DCI (Pohto & Paasonen, 1964; Bray, 1967) and propranolol (Bray, 1967; Pohto, 1967; Fukuda, 1968) but it is not prevented by the α-adrenergic blocking agent, phenoxybenzamine (Pohto & Paasonen, 1964) so it is clear that hypertrophy is caused by stimulation of β-adrenergic receptors.

Similarly, regarding induction of excretion of α-amylase, there is no difficulty in sorting out adrenergic from cholinergic effects (Pohto, 1968) nor in sorting out the α-adrenergic effects from the β-adrenergic effects of adrenaline (Batzri et al, 1971; Batzri & Selinger, 1973) but here the similarity ends. The effects of blocking agents are different. Although propranolol has some effect on reduction of secretion, there are compounds, such as ephedrine, which act as powerful sialogogues but have no effect on hypertrophy (Chan, 1964). This was

confirmed by Kirby et al (1969) who reported that modifications in the number of phenolic hydroxyl groups lead to differing responses. When both -OH groups are absent, the compound has no affect on the stimulation of DNA synthesis but is still capable of stimulating salivary gland excretion of α-amylase.

Both the d- and ℓ-stereoisomers of isoproterenol are active in stimulating DNA synthesis and α-amylase excretion in mouse salivary glands (Kirby et al, 1969) but ℓ-isoproterenol and the racemate (dℓ-isoproterenol) are far more effective in causing glycogenolysis in the liver. Also, ℓ- and dℓ-isoproterenol are considered much more effective in the induction of hypertrophy than d-isoproterenol (Novi & Baserga, 1971).

7. Dose Dependency

Workers in the field of experimental sialadenosis and induction of excretion in rodents, soon learned that it was not a case of "the bigger the dose of isoproterenol, the better". Although Selyé et al (1961) gave doses of 100 mg per day to rats in the 130 to 140 g weight group, daily doses exceeding 20 mg per day were found to be highly toxic, leading to high mortality rate and losses of body weight in survivors. In 1962, Schneyer reported that there was still a high death rate in 200 g rats treated with only 6 to 12 mg per day.

Only recently has pure ℓ-isoproterenol been available commercially, so dℓ-isoproterenol was used in all investigations

before 1972 and is still used in most studies. Pohto and Paasonen (1964) observed a gradual response in parotid gland enlargement with doses from 1 mg per kg to 100 mg per kg body weight and stated that small doses are more effective than large doses in producing hypertrophy. After a subcutaneous dose of 1 mg per kg daily for 7 days, the parotid glands in 150 g rats nearly doubled in weight. Baserga et al.,(1969) showed a correlation between the dose administered and the rate of DNA synthesis. In mouse salivary glands, an i.p. injection of 1 mmole (211 mg) of isoproterenol per kg body weight was found to produce a 10-fold increase in DNA synthesis. Guidotti et al.,(1972) studied the effect of varied doses of ℓ-isoproterenol on the concentration of cyclic AMP in mouse parotid gland. Ten minutes after injection, a maximum tissue concentration of cyclic AMP was obtained with 300 μmoles (74.2 mg) of ℓ-isoproterenol per kg body weight. Higher doses induced a smaller elevation in cyclic AMP levels. They attributed this inversion of the dose-response curve to toxic effects of isoproterenol (Guidotti et al.,1972). They also reported a correlation between the dose administered and the incorporation of ^{3}H-thymidine into DNA.

The excretion of α-amylase was also found to be dose dependent. Byrt (1966) reported 10 mg of dℓ-isoproterenol to cause secretion of 98% of the α-amylase present in salivary glands of rats weighing 140-170 g, but 1 mg was enough to deplete the glands of 84% of their α-amylase. Gaunce (1971) found that a dose of 5 mg

of dℓ-isoproterenol per 150 g rat (120 μmoles per kg body weight) resulted in complete excretion of α-amylase.

C. METABOLISM OF ISOPROTERENOL

After injecting rats with [^{3}H]-isoproterenol, Hertting (1964) reported that only three catabolites of isoproterenol are formed: these are 3-methoxy-isoproterenol, 3-methoxy-isoproterenol glucuronide and isoproterenol glucuronide. Because no deaminated catabolites are found in rat urine or bile, monoamine oxidase is not considered to play a role in the catabolism of circulating catecholamines: O-methylation is the principal pathway for their catabolism (Axelrod et al.,1958). O-methylation occurs mainly on the meta-hydroxyl group of catecholamines and the enzyme responsible is called catechol O-methyl transferase (COMT).

Salivary glands have a relatively high concentration of COMT (Axelrod et al.,1959). In the monkey, the only tissues with higher concentrations are the liver and pancreas. This fact may explain why isoproterenol is so rapidly metabolized. Baserga et al.,(1969) reported that if a dose of [^{3}H]-isoproterenol (800 μmoles per kg) was administered to mice, 51% remained unmetabolized in the salivary glands at the end of 30 min. When an inhibitor of COMT, pyrogallol, was injected prior to the isoproterenol, the percentage of unmetabolized isoproterenol was found to increase from 51% to 78% at 30 min. Using a

smaller dose of ℓ-isoproterenol (600 µmoles per kg), Guidotti et al. (1972) showed that the concentration of [^{3}H]-isoproterenol in mouse parotid gland reaches a maximum at 10 min after injection but that very little unmetabolized isoproterenol remains at the end of 40 min.

The enzyme, COMT, has an absolute requirement for Mg^{2+} or other divalent cation. The methyl group donor is S-adenosyl-methionine (SAM). Belleau (1966) suggested that the mechanism of action might involve the formation of a chelate, with two of the ligands supplied by the phenolic oxygens and the other two by SAM and the enzyme. Using the Mg^{2+} chelator, kojic acid (1×10^{-4}M), Mavrides (1964) showed that chelation of the Mg^{2+} does not lead to enzyme inhibition, at least at the concentration tested, so the validity of the proposed mechanism is doubtful.

Although COMT is thought by some to be localized almost entirely in the parenchymal cells of peripheral tissues (Jonason, 1969), there is evidence that it is located intraneuronally as well Alberici et al.,1965; Broch, 1971). Anderson and D'Iorio (1968) demonstrated multiple forms of COMT in rat liver. Later, multiple forms of COMT were found in the livers of humans and dogs as well (Axelrod & Vessell, 1970). Hertting (1964) has also shown that the binding and uptake of isoproterenol by various tissues is not similar to the binding and uptake of noradrenaline. No [^{3}H]-isoproterenol is present in any organ of the rat, except the kidney, within 2 hours after injection. Nevertheless, some claim to find radioactive isoproterenol in

the submandibular gland (Barka, 1970) and in the nuclei of salivary glands (Malamud & Baserga, 1967) still present at 24 hours after injection of a single does of ^{3}H-isoproterenol. Von Euler and Lishajko (1967) stated that nerve granules have the capacity to take up isoproterenol but are prevented by a barrier in the axon membrane. This barrier does not bar the entrance of noradrenaline and takes up adrenaline less readily than noradrenaline.

Following isoproterenol injection, monoamine oxidase has been reported to increase in salivary glands (Seifert, 1967; Mueller et al, 1968). On the other hand, Barka has stated that COMT is decreased at 26 hours after isoproterenol (Barka, 1970). Findings in this laboratory are the reverse; no change in monoamine oxidase is found at 4 hours after isoproterenol (Gaunce, 1971) but the COMT activity is much higher than controls at that time.

D. THE ADENYL CYCLASE SYSTEM

If isoproterenol is acting at β-adrenergic receptors (as the first messenger) to activate adenyl cyclase and produce cyclic AMP, then its effects ought to be simulated directly by the addition of cyclic AMP, the second messenger. Thus, many investigations have been initiated to prove that cyclic AMP produces the same responses as various hormones, but it is not that simple. Difficulties arise: in the first place, most

membranes are impermeable to cyclic AMP. If one homogenizes the tissue to overcome the first problem, a more serious problem emerges: the response of adenyl cyclase to hormones in broken cell preparations is extremely small compared to *in vivo* responses. Øye and Sutherland (1966) reported that the magnitude of the hormonal response varies inversely with the degree of homogenization. The release of proteolytic enzymes and depolarization of the membrane by homogenization may contribute to the problem as well.

The first problem was solved by Posternak *et al* (1962) who developed acyl derivatives of cyclic AMP which, by hydrophobic interactions with the lipid portion of membranes were better able to penetrate them. The mono- and dibutyryl derivatives are commonly used for this purpose, both *in vivo* and *in vitro*, using tissue slices, etc. Alternatively, the effects of a hormone at the membrane can be heightened by the use of an inhibitor of phosphodiesterase, the enzyme which hydrolyses cyclic AMP (Sutherland & Rall, 1958). Methyl xanthines, such as caffeine, aminophylline and theophylline, act as phosphodiesterase inhibitors.

In addition, methods for measuring adenyl cyclase activity in tissues have been not very reliable (Bar & Hechter, 1969). The outcome has been that, until the advent of better methods for measuring cyclic AMP, meaningful information could be obtained only from studies in which accumulation of cyclic AMP is

measured in intact cells.

1. Presence in salivary glands

The involvement of the adenyl cyclase system in the secretion of α-amylase by parotid gland slices was established by Bdolah and Schramm (1965) and Babad et al (1967). In 1970, Schramm and Naim reported that half of the adenyl cyclase activity of rat parotid is located in the 200 g fraction containing cell membranes and 15% is found in the microsomal fraction. The microsomal fraction is also shown to have a high affinity for cyclic AMP (Salomon & Schramm, 1970) and to contain more than 50% of the binding capacity of the particulate fraction. Following isoproterenol injection, Malamud (1969, 1972) reported increases in the adenyl cyclase activity at 2.5 and 20 min. Guidotti et al (1972) showed that within 1 min after injection of isoproterenol (600 μmoles per kg body weight), there is a significant elevation in the concentration of cyclic AMP in mouse parotid gland. It reaches a maximum 10 min after injection and returns almost to control levels between 1 and 2 hours after isoproterenol. In the presence of aminophylline, the concentration of cyclic AMP remains high for 80 min at least. Because of this finding they suggest that phosphodiesterase activity controls the intracellular level of cyclic AMP, although phosphodiesterase activity was found to be extremely low in salivary glands (Weiss et al, 1972).

2. Effects of cyclic AMP

That cyclic AMP is involved in salivary gland hypertrophy has been proven only indirectly. Wells (1967) reported enlargement of rat salivary glands after administration of theophylline. Effects on hyperplasia and DNA synthesis were reported after treatment with theophylline (Malamud, 1969) and aminophylline (Guidotti et al.,1972) caused increased incorporation of ^{3}H-thymidine.

In trying to establish cyclic AMP involvement in the secretion of α-amylase, Bdolah and Schramm (1965) and Babad et al.,(1967) used mono- and dibutyryl derivatives of cyclic AMP but encountered problems with spontaneous leakage. However, Malamud (1972) reported a decrease in α-amylase content of mouse parotid gland 20 min after injection of dibutyryl cyclic AMP.

Batzri et al.,(1971) reported that monobutyryl cyclic AMP causes a rapid and intensive secretion of α-amylase but adrenaline induces secretion of K^+ as well. They concluded that cyclic AMP mediates only the β-adrenergic effects of adrenaline and therefore K^+ release must be an α-adrenergic response. To test the hypothesis of Robison et al.,(1967) that α-adrenergic responses result from a decrease in cyclic AMP level, Batzri et al., (1973) tried to inhibit the release of K^+ by increasing the level of cyclic AMP. Because this response was not inhibited by increasing levels of cyclic AMP, they decided that α-adrenergic receptors do not operate through decreasing levels of cyclic AMP; instead, they suggest a system involving different degrees of stimulation of α- and β-receptors by catecholamines.

EXPERIMENTAL METHODS

PART I: GENERAL PROCEDURES

A. PREPARATION OF TISSUE HOMOGENATES

1. Treatment of rats

Male, Sprague-Dawley rats, weighing between 125 and 140 g were used in all experiments. They were cared for in individual polyethylene cages and fed Purina Laboratory Chow and tap water, ad libitum. Rats to be treated were fasted overnight and had access to water. Food was removed from the cages at 4 p.m. to allow at least 17 hours for repletion of salivary gland excretable proteins (Byrt, 1966). By 9 a.m. the glands were assumed to be in a fully-repleted state and the rats were injected intraperitoneally (i.p.) with one of the following, or a combination thereof:- i) 1 ml of isotonic saline (0.9%);

ii) 5 mg of dℓ-isoproterenol sulphate (M.W. 556; from Winthrop Laboratories) per 150 g body weight (120 μmoles per kg body weight) in 1 ml of isotonic saline;

iii) puromycin dihydrochloride (Nutritional Biochemicals Corp.), 100 mg per kg body weight, in divided doses of 40, 20, 20 and 20 mg per kg at hourly intervals;

iv) actinomycin D (Sigma Chemical Co.) in a single dose of 1 mg per kg body weight, dissolved first in 30% ethanol and diluted to 1 ml with isotonic saline.

Following treatment, rats were killed at intervals of 2 minutes to 6 hours by a blow to the head and exsanguination.

2. Dissection of salivary glands

The salivary glands were removed as rapidly as possible, using scissors, blunt tweezers and a magnifying dissecting lamp. An incision was made in the middle of neck and the skin pulled back to the base of the ear exposing salivary glands, their ducts and surrounding structures, such as the lacrimal gland. In rats of this size there is little or no adipose tissue, but the blood vessels, lymphatics and nerves lie within a framework of connective tissue which makes dissection of the delicate, three-lobed parotid gland very difficult, as others have reported (Schneyer & Schneyer, 1964; Gaunce, 1971). Its attachment at the base of the ear is freed first, then the connective tissue holding the other two lobes is gently disengaged and the common parotid duct severed. The entire parotid gland is then lifted out, placed on ice and any remaining connective tissue, blood vessels, lymph nodes, etc. carefully removed.

Dissection of the submandibular gland is relatively easy. The adventitia surrounding it and the sublingual gland is freed and the sublingual gland removed and discarded. Each pair of parotid and submandibular glands is weighed on a torsion balance and then placed in a glass homogenizing

tube (O.H. Johns Glass Co. Ltd.) with 5 ml of homogenizing medium per pair of glands.

B. TISSUE FRACTIONATION

1. Medium employed

Differential centrifugation to separate fractions depends on differences in the sedimentation coefficient existing between the particulate components of tissue homogenates. The sedimentation coefficient is a function of the size, shape and density of the particles but these physical properties are far from constant and depend, in a critical fashion, on the composition of the suspending medium (Beaufay & Berthet, 1963). Particles which are not separable in one type of medium become so in another, because of differences in osmolarity and tonicity and the resulting colloidal osmotic pressure, and also the concentration of specific agents which affect the degree of swelling of the particles (de Duve, 1963). This is particularly true for salivary gland homogenates, especially those of submandibular and sublingual, with mucous-synthesizing acini (the sublingual gland was always removed for this reason). The glycoproteins tend to cause agglutination and hence, entrapment of particles in a gel-like suspension. Many homogenizing media were tested and the following was found to give consistently good results, especially with parotid gland homogenate:-

Sucrose-tris-EDTA: 0.25 M sucrose; 0.01 M EDTA; and 0.02 M tris-HCl, pH 7.5.

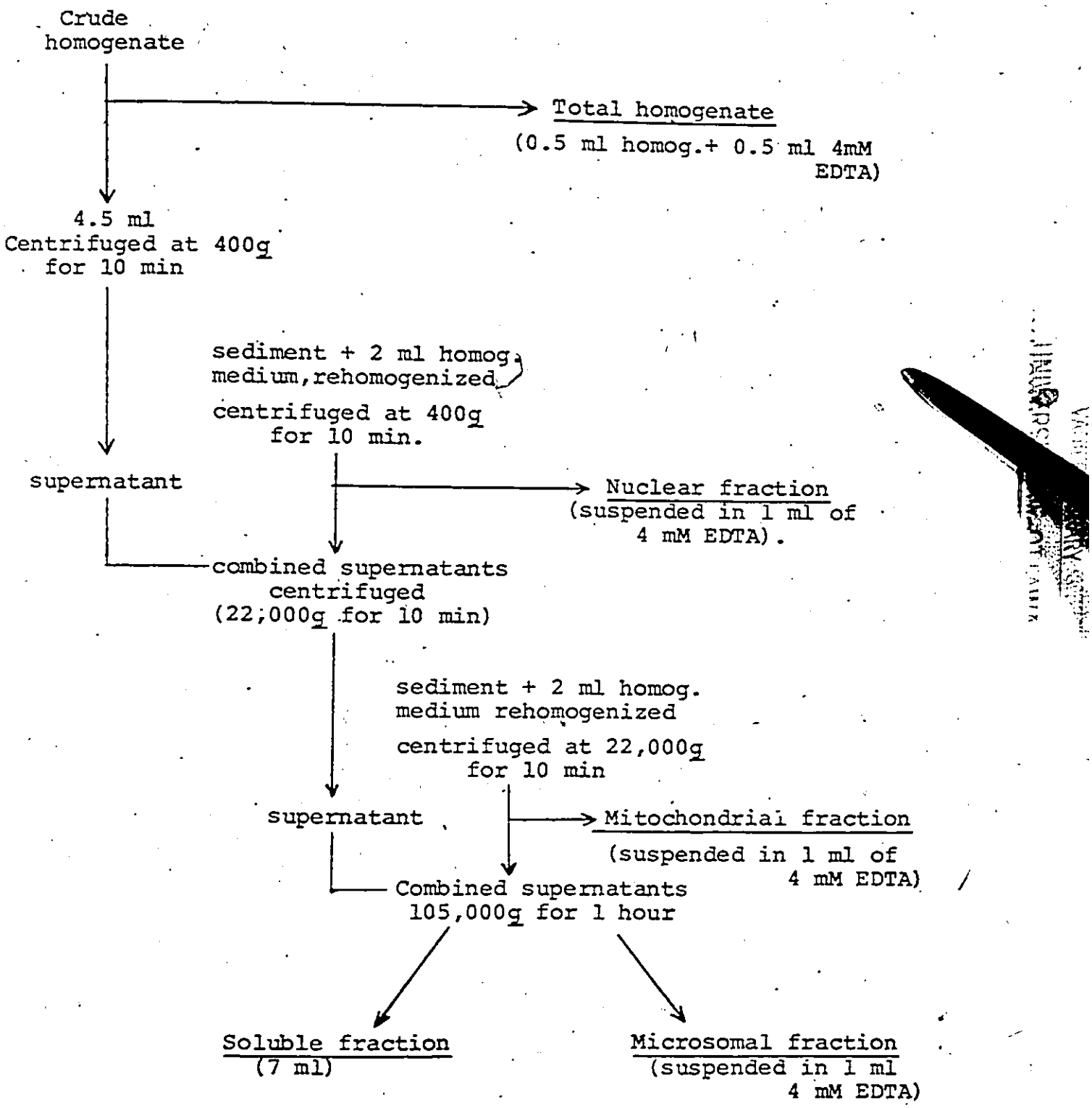
2. Homogenization

After mincing finely with small, pointed scissors, the glands were homogenized, using an Elvejhem-Potter type homogenizer (Cenco) equipped with a teflon pestle giving clearance of 0.15 mm. To maintain the integrity of the intracellular organelles, the amount of homogenization was limited to 15 strokes. Usually 10% of the homogenate was diluted to 1 ml per pair of glands, with distilled water or 4 mM EDTA, and set aside for analysis of enzyme activity and protein determination in the crude homogenate, as shown in Figure 6. The other 90% was fractionated by differential centrifugation, as outlined below.

3. Fractionation of homogenate

The scheme used for fractionation of the homogenate is shown in Figure 6. The nuclear fraction was obtained by centrifuging at 400 g for 10 min using a Sorvall automatic refrigerated centrifuge (Model RC2B) at 1°C. The fraction which sedimented was washed in 2 ml of homogenizing medium (or twice in 1 ml of medium) and rehomogenized. From the combined supernatants the crude mitochondrial fraction was spun down at 22,000 g for 10 min and also washed in 2 ml of homogenizing medium. The supernatants were again combined and further centrifuged in a Beckman Ultracentrifuge, Model L2-65B, at 100,000 g for 60 min to obtain the microsomal and soluble fractions. All particulate fractions were suspended in 1 ml of water or 4 mM EDTA.

Figure 6. Fractionation of rat salivary gland homogenate



Although the fractions are termed "nuclear", "mitochondrial", "microsomal" and "soluble" they are not to be construed as being composed of homogeneous organelles; they are intended as highly reproducible fractions which contain the organelles for which they are named along with other particles having the same sedimentation rate. In a given population of cell particles there may be overlapping of different particles in any type of centrifugation separation, however, one can still obtain an enormous amount of information regarding localization of enzymes and their relationship to known functions of the tissue being studied.

4. Validity of the fractions

According to de Duve one can only state that he has been working with a pure fraction if the organelles, have been identified by electron microscopy. However, he states that even then everything seen cannot be identified and of course adsorbed enzymes and cytoplasmic contaminants do not show up. Previous work in this laboratory has also shown that many of the marker enzymes, such as glucose-6-phosphatase and UDP-glucuronyl transferase, which are normally used to identify fractions in other tissues, are not present in salivary glands (Gaunce, 1971). Cytochrome oxidase is one of the few present and it is localized almost entirely in the mitochondrion. In the other particulate fractions, the

amount of cytochrome oxidase was found to vary directly with the amount of protein present in the fraction. In Table II from the thesis of Gaunce, (1971) we find that normally from 10 to 13% of the protein is present in the nuclear fraction and in that case 80% of the cytochrome oxidase is present in the mitochondrion; but if particles are trapped in the nuclear fraction, its protein content rises to over 20% and then 75% of the cytochrome oxidase is there also and only 21% in the mitochondrial fraction. Therefore, in the present experiments the amount of protein in each fraction is the criterion used to judge whether uniform fractions are obtained. The zymogen granules have been found to be completely lysed by the homogenizing medium (Gaunce, 1971), so that their contents are included in the soluble fraction. Thus, the protein distribution for normal, starved rats is considered typical if it is within 10% of the following:

Nuclear fraction	10%
Mitochondrial fraction	12%
Microsomal fraction	8%
Soluble fraction	70%

During and following excretion of salivary enzymes, this distribution no longer applies. Because the zymogen granules have been lysed, the decrease in protein due to excretion is reflected mainly in the protein content of the soluble fraction.

TABLE II

RELATION OF PROTEIN DISTRIBUTION TO CYTOCHROME OXIDASE IN SUBCELLULAR FRACTIONS AFTER DIFFERENTIAL CENTRIFUGATION**

	Per cent distribution*			
	Control		Sucrose-KCL-EDTA medium	
	Cytochrome oxidase	Protein	Cytochrome oxidase	Protein
Parotid				
Nuclear	75	24	13	13
Mitochondrial	21	8	80	15
Soluble†	4	68	7	72
Submaxillary				
Nuclear	52	20	10	10
Mitochondria	44	11	84	15
Soluble†	3	69	6	75

* Activity in each fraction expressed as a per cent of the sum of activities in all fractions.

† Mitochondrial supernatant: Contains microsomal and soluble fractions.

** From thesis, Control of Salivary Gland Enzymes (Gaunce, 1971) p. 278.

C. PROTEIN DETERMINATION

The use of Folin phenol reagent in protein determination was first reported by Wu (1922). The method was later revised by Lowry et al, (1951). It is a sensitive, colorimetric method in which the protein present reacts first with copper in alkaline solution and then the copper-treated protein reduces the phosphomolybdic-phosphotungstic reagent.

1. Reagents

A. 1% $CuSO_4$

B. 2% Na or K tartrate

C. 2% Na_2CO_3 in 0.1 N NaOH

D. 1 N Phenol reagent (Fisher Scientific Co.)

2. Solution A (freshly prepared)

To 50 ml of Reagent C, add 0.5 ml each of A and B.

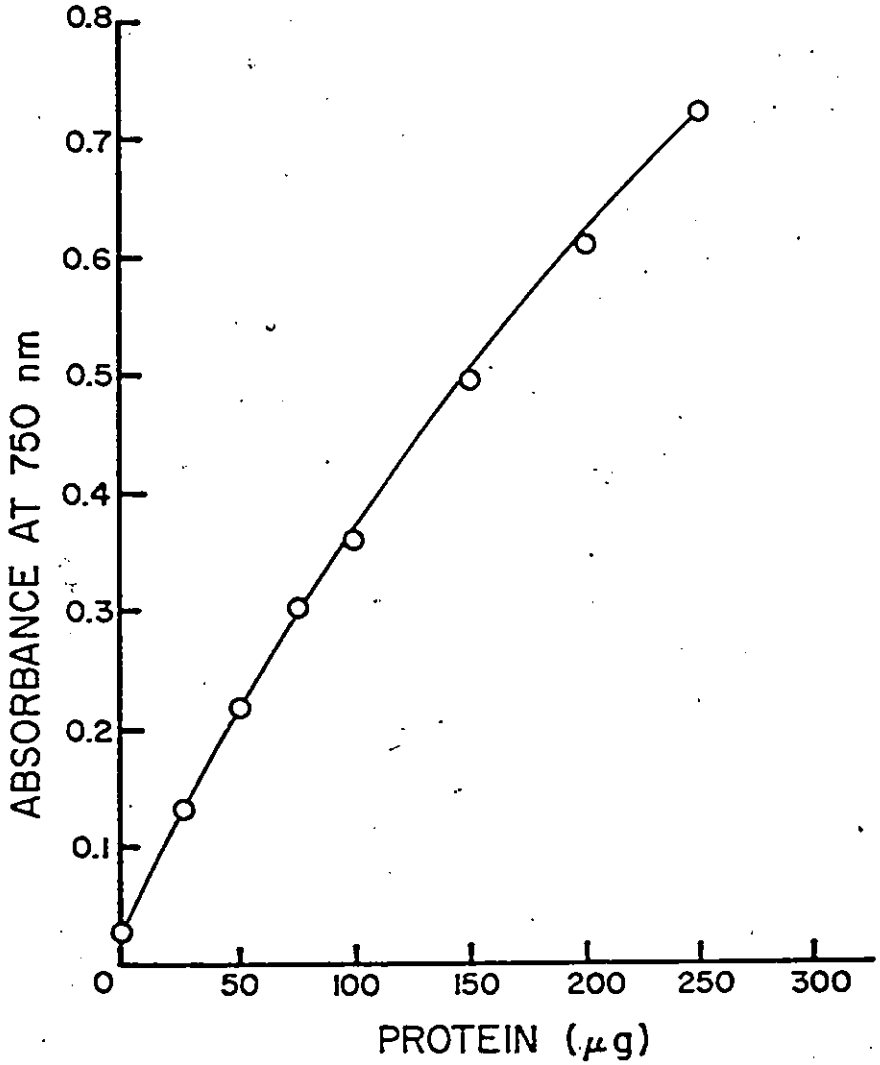
3. Protein standards

A solution of crystalline bovine albumin (Sigma Chemical Co.) containing 500µg per ml was prepared; aliquots of 50 to 250µl were placed in separate tubes and the volume brought to 1 ml with distilled H_2O; a blank tube contained 1 ml of H_2O only.

4. Method

Aliquots of homogenates and fractions (usually 25µl) were diluted to 1 ml. 5 ml of Solution A was added to all tubes (samples and standards) and left at r.t. for 10 min. Then, 0.5 ml of Reagent D was added, mixed thoroughly and after allowing 30 min for colour to develop, the absorbance was read on a Beckman Spectrophotometer, Acta III (wavelength 750 nm). A calibration curve was prepared from readings for standards (Figure 7) and the protein content of samples determined.

FIGURE 7. CALIBRATION CURVE FOR PROTEIN DETERMINATIONS



D. RADIOACTIVE ANALYSIS

1. Scintillator fluids used

Samples to be analysed for ^{14}C or ^{32}P (weak and strong β-particle emitters) were placed in polyethylene vials with screw caps (O.H. Johns Scientific or New England Nuclear Co.) and one of the following scintillator fluids was added, as indicated:-

a) Toluene-PPO: Omnifluor (New England Nuclear Co.) is a crystalline mixture of 98% 2,5-diphenyloxazole (PPO) and 2% Bis-MSB, a patented secondary scintillator (spectrum shifter). Four (4) g of Omnifluor was added to 1 litre of scintillation-grade toluene.

b) Aquasol (New England Nuclear Co.): A xylene-containing liquid scintillation counting solution which permits efficient counting of protein dissolved in NaOH, when encountered in these studies.

2. Apparatus

All radioactive samples were counted in a Nuclear Chicago Corp. temperature-controlled Liquid Scintillation System, Mark I. This is a three-channel scintillation counter, geometry optimized, with external standardization which can be used optionally.

3. Corrections applied to observed counts

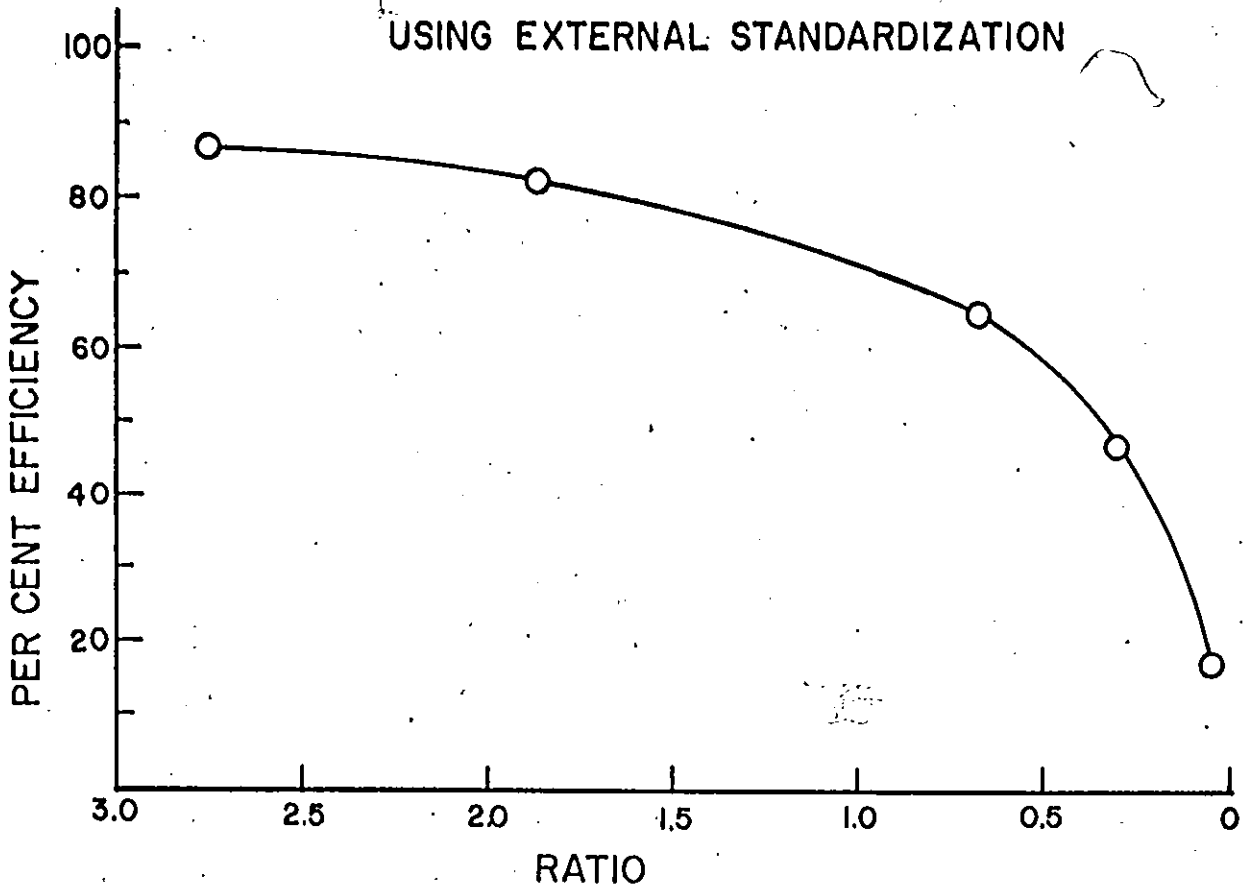
a) Quenching corrections: Substances which cause quenching in the samples prevent some of the photons of light from

reaching the photomultiplier tubes. These substances include, any coloured compound, hydrogen ions, water, organic substances, etc.

The efficiency of counting was determined using the external standardization method. A barium-133 source is provided for this purpose and two channels are used; one is set for the balance point using the least quenched tritium sample and the other is adjusted to count the balance point for the barium source, using the same unquenched tritium standard. The ratio of counts per minute (c.p.m.) in the barium channel to the c.p.m. for barium in the tritium channel, changes with the degree of quenching in the sample. As the sample quenching increases, the barium count rate decreases inversely. A calibration curve relating counting efficiency for the isotope of interest to the ratio of barium count rate in two channels is constructed by counting a series of quenched standards of the isotope of interest. From the known disintegrations per min (d.p.m.) of the standards, the per cent efficiency is plotted (efficiency = $\frac{\text{c.p.m.}}{\text{d.p.m.}}$). For example, a set of ^{14}C quenched standards give a calibration curve as shown in Figure 8. The per cent efficiency is located for each sample and the d.p.m. calculated.

b) Radioactive decay (for ^{32}P samples only): This correction is negligible for ^{14}C because its half-life is over 5,000 years; but for ^{32}P, with a half-life of only 14.3 days, it is necessary to extrapolate the observed counts back to the assay date.

Figure 8. QUENCH CORRECTION CURVE FOR ^{14}C STANDARDS USING EXTERNAL STANDARDIZATION



c) Background: A blank sample was counted with each set of samples to determine background counts and to check for contamination. The background counts (usually around 20 c.p.m.) were subtracted from the total c.p.m. for each sample.

4. Statistical accuracy

Because radioactive decay and emission are random events, the longer the counting time, the closer one approaches the actual mean count rate. When n = the c.p.m. observed, the standard deviation, $\sigma = \sqrt{n}$. The relative standard deviation $= \sqrt{n} \times \frac{100}{n}$ or $\frac{100}{\sqrt{n}}$. In order to work to 1% standard deviation i.e. for $\frac{100}{\sqrt{n}} = 1$, n must equal 10,000. Therefore, each sample was counted long enough so that at least 10,000 counts were recorded, in order to achieve a standard deviation equal to ±1%.

E. STATISTICAL ANALYSIS OF DATA

Results are given as mean value ± standard error of the mean (S.E.M.) where possible. Student's t-test is applied when necessary to determine if differences are significant. The "P" value of significance is considered at the 0.05 level of confidence.

PART II: ENZYME ASSAYS

A. CATECHOL O-METHYL TRANSFERASE (EC 2.1.1.6)

1. Methods used by others

The radioactive assay for catechol O-methyl transferase (COMT) used herein, was first described by Pellerin and D'Iorio (1958). L-methionine-methyl-^{14}C was used as methyl donor and ten different catechol acids were tested as substrates. The methoxy derivative was extracted with ethyl acetate. Because 3,4,-dihydroxybenzoic acid (Protochatechuic acid) was the most active substrate and did not participate in any secondary reaction in the presence of a crude enzymic preparation, it was the substrate chosen for this method of measurement of O-methyl transferase, as published by D'Iorio (1961).

A fluorimetric procedure was described by Axelrod and Tomchick (1958). Adrenaline bitartrate served as substrate and S-adenosylmethionine (SAM) as the methyl donor. The metanephrine formed was measured spectrofluorimetrically. In 1959, a radioactive method, using ^{3}H-adrenaline as substrate, was described by Axelrod et al, (1959). The enzymically formed ^{3}H-metanephrine was extracted by an isoamyl alcohol-toluene solvent system and determined by scintillation counting. Both procedures were subject to potential variability because metanephrine was not quantitatively extracted by the solvent system used.

The method of D'Iorio (1961) was slightly modified by McCaman (1965) who used [^{14}C-methyl]-SAM as the methyl donor in a micro-determination with a total volume of 25 µl. This micromethod was tested by us as described, using 0.4 ml polyethylene tubes (Arthur H. Thomas Co.). The extraction procedure with ethyl acetate requires thorough mixing which is difficult to achieve when using such tiny plastic tubes. Therefore, the same method was adapted to a larger volume (125 µl) in 4 ml glass tubes. The efficiency of the method improved considerably.

2. COMT Assay

The incubation mixture contained the following constituents at the indicated final concentration: potassium phosphate, 0.08 M (pH 7.8); $MgCl_2$, 5 x 10^{-3} M; 3,4-dihydroxybenzoic acid (K. & K. Laboratories), 1 x 10^{-3} M; and S-adenosyl-L-methionine-methyl-C^{14} (New England Nuclear), 6 x 10^{-5} M. The [^{14}C-methyl]-SAM had a specific activity of 52 mCi/mM to start with but after dilution with unlabelled SAM the specific activity was approximately 65,000 d.p.m. per nanomole.

3. Experimental procedure

COMT activity was assayed in the homogenate and fractions of parotid and submandibular glands of normal, fasted rats and of those injected with isoproterenol for periods of

10 min, 30 min, 1 hour, 2 hours, 3 hours and 6 hours. The effect of the protein synthesis inhibitor, puromycin, was also tested, along with isoproterenol for 2 hours in order to see if the increases in enzyme activity are due to synthesis of new enzyme protein.

Glass test tubes were placed on ice. 100 µl of the prepared incubation solution was placed in each tube, and 25 µl of water (for the blanks) or enzyme solution was added to each. After mixing, the tubes were placed in a water bath at 38°C with shaking, for 30 min. The reaction was terminated by addition of 15 µl of 3 N HCl to each tube, and the tubes were placed on ice. 0.5 ml of ethyl acetate was added and thoroughly mixed to extract the 3-methoxy-4-hydroxybenzoic acid. After centrifuging at 10,000 g for 10 min to separate the phases, a 50 µl portion of the ethyl acetate was removed and placed in counting vials. 15 ml of toluene-PPO scintillator fluid was added to each vial and the radioactivity determined as described under Methods, I-D. Enzyme activity was calculated on the basis of the known specific activity of the [^{14}C-methyl]-SAM and was expressed as nmoles of labelled product formed per gland (or per g wet weight) per 30 min. The concentration of SAM used (60 µM) gave optimal activity. Higher concentrations were found to be completely inhibitory (McCaman, 1965).

B. ADENYL CYCLASE

1. Methods used by others

Several approaches have been used for the measurement of cyclic AMP, the product formed during adenyl cyclase activity. They include:

a) Enzyme activation: Rall and Sutherland (1958) originally described a method for measuring cyclic AMP based on the activation of the phosphorylase system of liver by cyclic AMP. This method was modified by Butcher et al, (1965) who isolated cyclic AMP from other tissue nucleotides by column chromatography on Dowex ion-exchange resin columns, a procedure requiring about 2 days.

b) Enzyme conversion with direct product analysis: Cyclic nucleotide phosphodiesterase was used to convert cyclic AMP to 5'-AMP which was then converted to ADP with adenylate kinase (Turtle & Kipnis, 1967) or to ATP (Aurbach & Houston, 1968).

c) Enzyme conversion coupled to cycling systems for product analysis: The original enzyme cycling technique was described by Breckenridge (1964) and was modified by Goldberg et al, (1969). In the enzyme cycling systems the amount of ADP or ATP formed was magnified 1000- to 5000-fold and the final product formed (e.g. glucose-6-phosphate) was then measured by fluorimetric or spectrophotometric techniques.

d) Radioisotope displacement: This type of assay is based on the competition of unlabelled cyclic AMP in the tissue extract with added tritiated cyclic AMP for conversion to the 5'-nucleoside monophosphate by specific phosphodiesterase preparations (Brooker et al, 1968).

e) Chemical assays: Several chemical assays have been described for the measurement of cyclic AMP, for example that of Pauk and Reddy (1967); Krishna et al,(1968); and Bradham and Wooley (1964). In the first, cyclic AMP is isolated by ion exchange chromatography and then acetylated at the 2'-O position with ^{3}H-acetic anhydride using ^{14}C-cyclic AMP as an internal standard to determine both conversion and recovery. The high cost of labelled acetic anhydride, the insensitivity of the method and the multiple isolation and chemical procedures involved, limited the usefulness of this assay.

Most of the above-mentioned methods were either time-consuming, insensitive, expensive, or all three. In addition, even widely-employed methods, such as that of Krishna et al, (1968), were found to be unreliable for low concentrations of cyclic AMP (Bar & Hechter, 1969). In some of the methods,hypoxanthine was being measured instead of cyclic AMP and the recovery after removing other nucleotides by $ZnSO_4$-$Ba(OH)_2$ treatment was invariably low. ATPase competed with cyclic AMP for the ATP in the tissue and this problem was overcome, only partially, by use of an ATP-regenerating system, such as

phosphoenolpyruvate (PEP) and pyruvate kinase. In many cases, adenylate kinase (myokinase) was needed as well to maintain the ATP concentration (Dousa & Rychlik, 1968). Alpha-labelled ^{32}P-ATP prevented the interference caused by other radioactive break-down products of ATP formed from uniformly-labelled ^{14}C-ATP or ^{3}H-ATP.

Newer methods based on saturation analysis and radioimmunoassays appear to be more promising:

f) Saturation analysis: This method depends on the capacity of protein fractions containing protein kinase to bind the cyclic AMP (Gilman, 1970; Walton & Garren, 1970). Even this method is susceptible to error if the tissue extract contains substances which interfere with the binding reaction. Weller et al (1972) have therefore modified the Gilman method.

g) Radioimmunoassays: The radioimmunoassay for cyclic nucleotides is similar to the radioimmunoassay techniques developed for peptide hormones in 1960 and is based upon competition of the cyclic nucleotide with an isotopically labelled derivative of the cyclic nucleotide for binding sites on an antibody, specific for the cyclic nucleotide. The method developed by Steiner et al (1969) eliminates the need for chromatographic separation of cyclic AMP from other tissue nucleotides.

2. Procedure used herein

Two methods were used for measuring adenyl cyclase activity.

The first involved the use of [α-^{32}P]-ATP or ^{14}C-ATP and separation of the labelled product, cyclic AMP, by thin layer chromatography (TLC) by a method adapted from that of Randerath and Randerath (1964) for separation of other nucleotides.

The second method involved the activation of a partially purified protein kinase from parotid gland which was sensitive to picomolar amounts of cyclic AMP. To measure the activation resulting from tissue cyclic AMP, phosphodiesterase must be rapidly destroyed. Methods based on this principle are described by Kuo and Greengard (1970), Butcher (1971) and Wastila et al (1971).

a) Adenyl cyclase assay: For measuring reaction rates, incubation volume began at 1.0 ml and 0.1 ml aliquots were removed at 5 min intervals. For individual assays, volumes of 0.4 ml were usually employed. The assay system contained: 40 mM Tris-HCl, pH 7.5; 10 mM theophylline (or caffeine); 5 mM $MgCl_2$; 3mM ATP and [α-^{32}P]-ATP or ^{14}C-ATP, 0.5 - 2.0 µCi; and 0.1% bovine serum albumin. In addition, an ATP-regenerating system was included, either 5 mM PEP and pyruvate kinase, 0.05 mg/ml; or 10 mM phosphocreatine and creatine phosphokinase, 18 units; and finally, myokinase, 75 units, was used in later experiments. The following solutions were included, where specified, to test for effects: 10 mM sodium fluoride; 5 mM EGTA; 5 mM $CaCl_2$; 50 µM isoproterenol and 50 µM noradrenaline. The mixture was equilibrated at 37°C and the reaction was started by addition of suitably diluted

enzyme and allowed to incubate in a water bath, with shaking, for 8 min. The reaction was terminated by placing the tubes in a boiling water bath for 3 min after addition of 50 µl of a solution containing 5 mM ATP and 5 mM cyclic AMP, as carriers. The tubes were centrifuged at 9,000 g for 5 min to remove denatured protein.

b) Thin layer chromatography (TLC) of nucleotides

10 µl samples of the clear supernatant were applied to polyethyleneimine (PEI), 20 x 20 cm sheets (produced by Macherey-Nagel & Co. and supplied by Brinkmann Instruments Co.). From 13 to 18 samples were applied per sheet, at a distance of 2.5 cm from the bottom, using disposable micropipets (Scientific Products). A mixture of standards containing ATP, AMP, adenine, adenosine and cyclic AMP was also applied (Figure 9). When completely dry, the sheets were washed in anhydrous reagent grade methanol for 5 min to remove interfering electrolytes, etc. After 15 min the sheets were developed in glass chromatography tanks by stepwise elution; first in distilled water for 2 cm, then in 0.3 M lithium chloride until the front reached 8.5 cm from origin, and finally in 1.0 M lithium chloride for the last 1.5 cm (total distance from origin to front = 10 cm). The developed chromatograms, when dry, were visualized under ultraviolet light. The absorbing spots were outlined with a soft pencil. Then the cyclic AMP, ATP and other spots, localized in relation to the standards, were cut out, placed in 15 ml of toluene-PPO

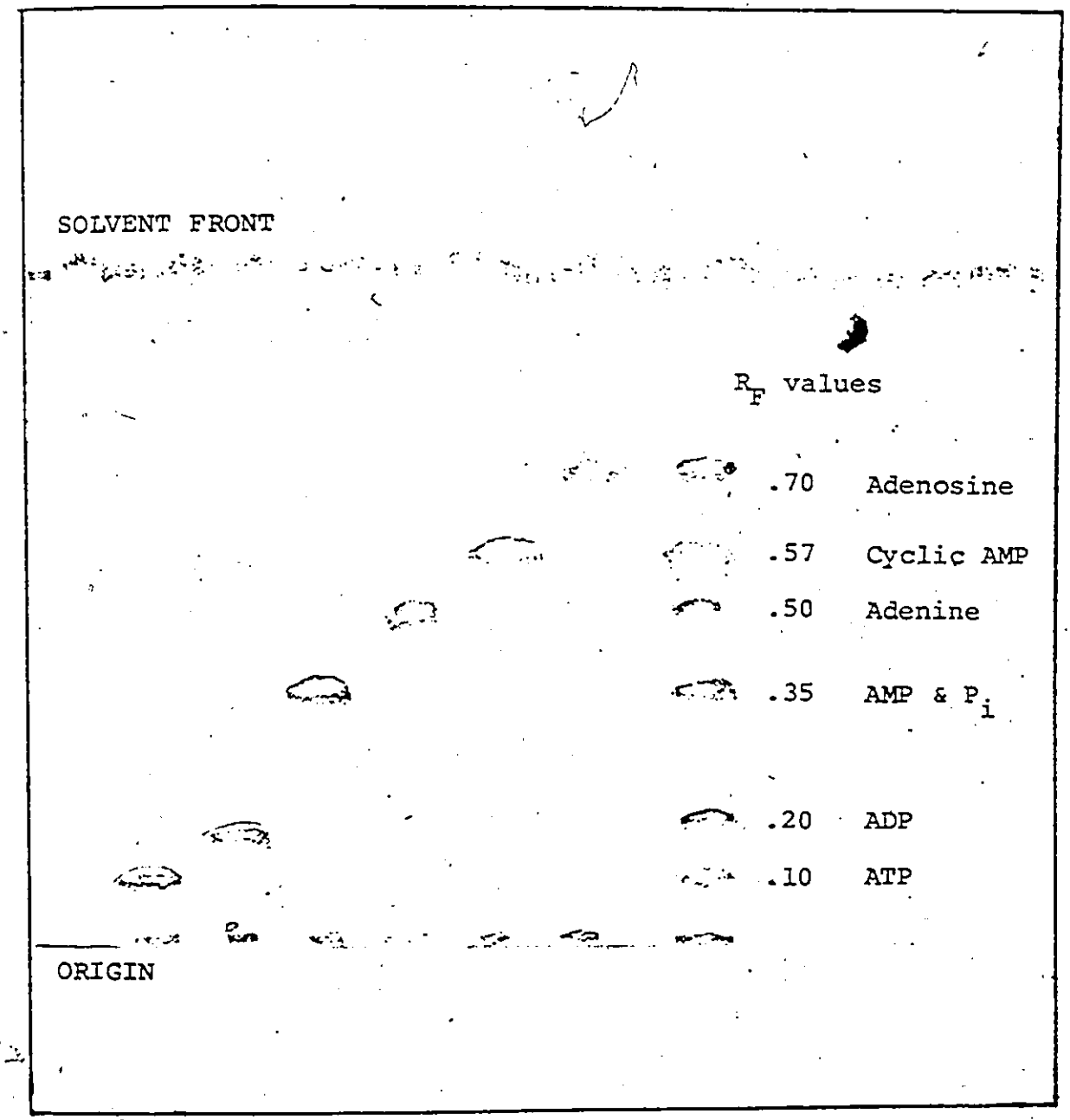


Figure 9. Chromatography of nucleotides on thin layers of polyethyleneimine (PEI) cellulose.

and counted as described under Methods (I-D). The per cent conversion of labelled ATP to cyclic AMP was calculated from the d.p.m. in the ATP and cyclic AMP spots and the amount of cyclic AMP formed was calculated from the specific activity of the ATP. All assays were carried out in triplicate with appropriate blanks.

Carrier cyclic AMP was added to all samples, as mentioned in assay description. When [^{3}H]-cyclic AMP was added to the carrier, over 90% of the radioactivity was recovered in the cyclic AMP spot.

c) Tissue levels of cyclic AMP

Tissue levels of cyclic AMP were measured in rat parotid gland before and after injection of 120 µmoles of dℓ-isoproterenol per kg body weight. At intervals of 2 min to 4 hours after the intraperitoneal injection, samples of tissue were quickly removed, weighed and immediately homogenized in 0.5 ml of 10% TCA in order to destroy any phosphodiesterase activity. Dissection of the entire gland was omitted for same reason. After standing at 0°C for 10 min, the protein was spun down at 48,000 g for 10 min. The supernatant was neutralized with 1 M Tris, then freeze-dried (Virtis) and redissolved in 0.1 ml H_2O. This solution was then used in protein kinase assays (described below) to activate partially purified protein kinase from parotid gland, prepared as described in Part III

C. PROTEIN KINASE

The assay used for protein kinase is quite similar to that originally described by Walsh et al. (1968) and modified slightly by Kuo et al. (1970).

1. Protein kinase assay

Protein kinase activity was determined in 0.25 ml of an incubation mixture containing 50 mM sodium glycerophosphate, pH 6.5; 10 mM magnesium acetate; 10 mM sodium fluoride; 2 mM theophylline; 0.3 mM EGTA; 0.2 mM ATP; [γ-^{32}P]-ATP, 0.5 µCi. In addition, extra ATP (1-2 mM); 5 µM cyclic AMP; 0.2 mg histone, calf thymus, type 2 (Sigma) or 0.6 mg casein, vitamin-free (Fisher Scientific Co.) were added to test for effects, where indicated. To initiate the reaction, 70 µg (approx.) of enzyme protein were added to each tube and tubes were incubated, with shaking, in a 30°C water bath for 7 min. The reaction was terminated by adding 5 ml of 10% TCA and 0.2 ml of 0.6% bovine serum albumin as carrier protein, and allowed to stand at 0°C for at least 20 min. The tubes were then centrifuged at 8,000 g for 10 min to separate the phosphorylated proteins which were washed and treated as described below.

2. Methods for washing phosphorylated proteins

Cyclic AMP-dependent protein kinases have been shown by many to catalyze the formation of phosphoester bonds with serine and threonine side-chains of proteins (Langan, 1968; Turkington & Riddle, 1969; Johnson et al.,1971). To distinguish this form of phosphorylated protein from the acyl phosphate intermedate formed by ATPase (Hokin et al.,1965), it was deemed necessary to include treatment with NaOH during the washing procedure in order to hydrolyze acyl phosphate bonds and remove any adsorbed phosphates.

Two methods for washing the phosphorylated proteins were in general use. Method (a) includes the NaOH treatment and was used in many experiments described herein but was found to be tedious, time-consuming and protein recovery was variable. A modification of methods (a) and (b) was developed which is less time-consuming and more efficient and reproducible.

a) Stirring, centrifugation and decantation: This method was described by Delange et al.(1968). Following centrifugation, the supernatant is removed by aspiration and the TCA-insoluble protein dissolved in 1.0 ml of 0.1 N NaOH and the protein reprecipitated with 10% TCA. Centrifugation and decantation are repeated. The pellets are then washed with 5% TCA and again collected by centrifugation. The well-drained pellets are then dissolved, usually in a small amount of N NaOH (Miyamoto et al.,(1969) and the radioactivity counted.

b) Filter paper disc method: The use of filter paper discs in separating phosphorylated protein has been described by many, e.g. Mans and Novelli (1961); Langan (1968); Butcher (1971); etc. In most cases the TCA-insoluble material is collected on small squares of fine (0.45 micron) filter paper (or commercially available discs in assorted sizes). The papers are washed in beakers of TCA with the aid of a mixer. For example, Reimann et al.,(1971) pipet 50 µl of the reaction mixture onto squares (2 x 2 cm) of Whatman No. 31ET chromatography paper. The papers are then washed in cold 10% TCA for 30 min, cold 5% TCA for 10 min and twice in 5% TCA for 10 min at r.t. using 5 to 10 ml of TCA per paper in each wash. Then the papers are washed briefly in ethanol, rinsed with ether, dried and transferred to liquid scintillation vials.

c) Method developed herein: After centrifuging to collect the TCA-insoluble protein, the supernatant was decanted and the pellet dissolved in 0.2 ml of 1 N NaOH and immediately reprecipitated with 10% TCA. After standing at 0°C for 15 min, the solution was filtered through a 0.45 µ

cellulose filter disc, 25 mm diameter (R & B Filters, Sartorious or Millipore HAWP). The filter disc was supported by a filter holder funnel with polypropylene barrel (Canlab). After washing, the discs were placed in counting vials, 15 ml of toluene-PPO were added and the ^{32}P incorporated into the protein counted in the Liquid Scintillation Counter, as described in Methods I-D.

When method (a) was used herein, the final protein dissolution was in 0.1 ml of 1 N NaOH. The dissolved protein was then shaken with 10 ml of Aquasol and counted (Methods I-D).

PART III: PARTIAL PURIFICATION OF PROTEIN KINASE

The usual enzyme purification steps were modified somewhat because of aggregation and adsorbing effects of salivary gland glycoproteins, especially in submandibular glands. The acid-precipitation step was omitted. From reports in the literature it was believed, at first, that all activity was present in the soluble fraction, so purification attempts were performed mainly with the 27,000 g soluble fraction. All procedures were carried out at 4°C.

1. Differential centrifugation

The crude homogenate was centrifuged at 27,000 g for 30 min and the supernatant tested for protein kinase activity or further purified. The particulate fraction was found to contain much activity also; hence the decision to test subcellular fractions.

2. Ion-exchange chromatography

The anion-exchanger, diethylaminoethyl (DEAE) cellulose (Sigma Chemical Co.) was pretreated in the conventional way, as described by Peterson and Sober (1962): 10 g were stirred into 500 ml H_2O and after settling for 15 min the supernatant and fine

particles were decanted. This was repeated twice. After resuspending again in water, the cellulose was filtered with suction, using a Buchner funnel. The filter cake was activated by resuspending in 500 ml of 0.5 N KOH; another 500 ml of water was added and the suspension refiltered with suction. The filter cake was then resuspended and washed until free of alkali. Following this it was equilibrated with 5 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. A fairly dilute suspension of the cellulose and buffer was used to pack a 0.9 x 15.0 cm column (Pharmacia) as shown in Figure 10. The buffer flowed at about 30 ml per hour while the cellulose settled and the suspension in the column was periodically replenished without allowing the top of the packed cellulose to become disturbed or to dry out. When the level of cellulose was within 3 cm of the top of the column, the top was put in place and the flow valve adjusted for continual replacement of buffer from a reservoir placed above the level of the column.

The protein sample, about 20 mg in approx. 5 ml (previously dialysed in the same buffer) was applied without disturbing the top of the cellulose. Fractions were collected in 8.5 ml aliquots using a fraction collector (LKB 7000 UltroRac).

The proteins were eluted with a linearly increasing concentration gradient of NaCl (0 to 1.0 M) using two flasks, one mounted above the other, with a connection from the bottom of the top vessel (which contained the higher concentration) to

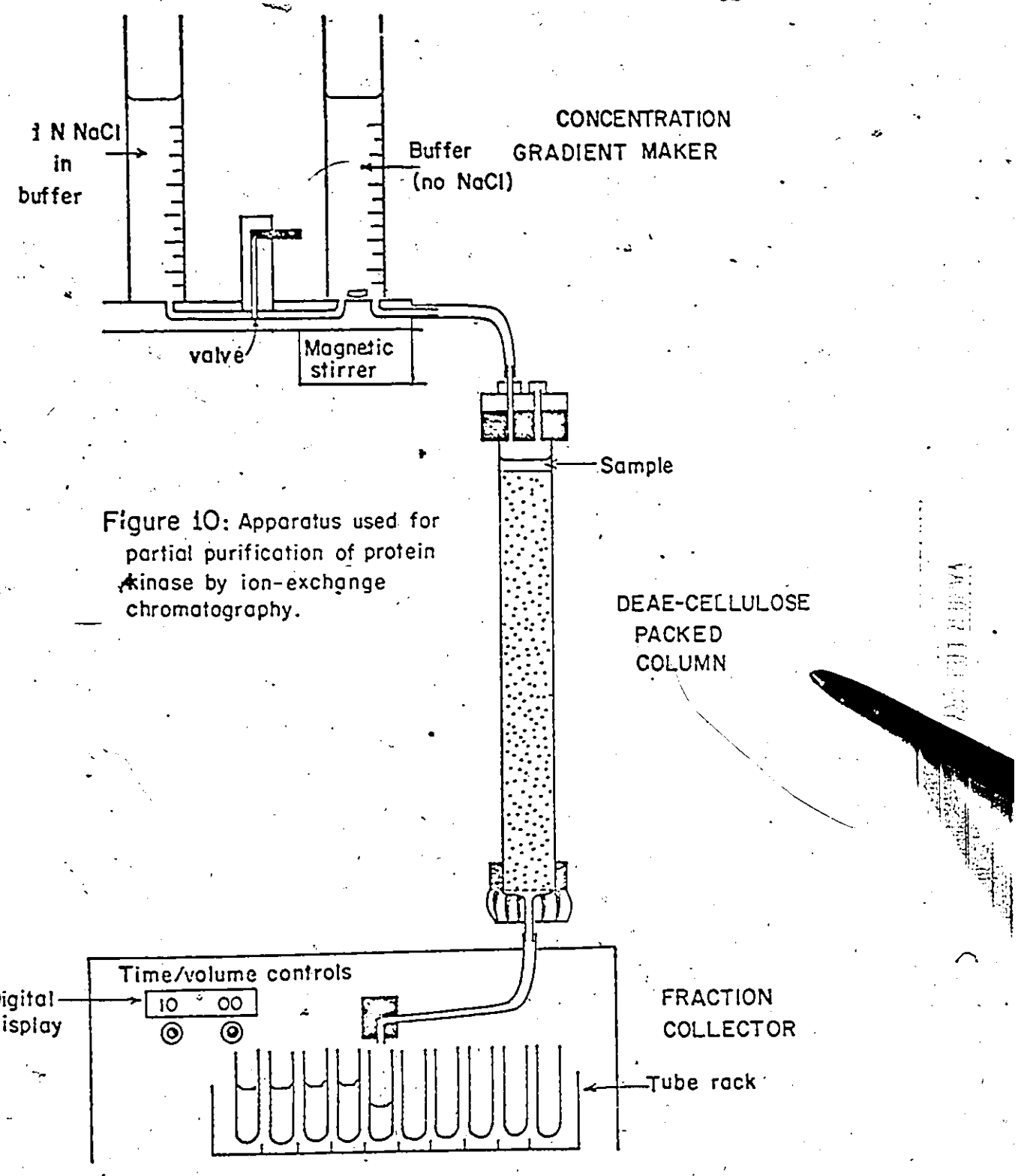


Figure 10: Apparatus used for partial purification of protein kinase by ion-exchange chromatography.

to the lower one (filled to same level with starting buffer). Liquid leaving the lower flask, the contents of which are stirred continuously with a magnetic stirrer (Fisher Scientific Co.), is automatically replaced from the upper flask, causing the concentration to increase as follows:

$$C = C_2 - D\, e^{-k}$$

where C is the concentration of the solution being withdrawn; C_2 is the concentration in the upper vessel; D is the difference between the initial concentration of the upper and lower solutions; and k is the ratio of the volume removed (up to that point) to the fixed volume of the solution in the lower vessel. The gradient produced by this set-up is slightly curved, but, in later experiments a device for providing an absolutely linear concentration gradient (Chrismac plastic fabrications) was utilized, as shown in Figure 10, and proved easier to manipulate. Equally good separation of proteins was obtained by either method of preparing the gradient. The protein in the fractions was measured by the absorbance at 280 nm on the Beckman Spectrophotometer, Acta III.

3. Salt fractionation

Fractions from the column, which contained protein peaks having protein kinase activity, were treated with solid ammonium sulphate (Schwarz Mann) to a concentration of 35% (w/v). After stirring for 30 min, the precipitate was collected by

centrifugation and dissolved in 5 mM potassium phosphate buffer containing 2 mM EDTA. The resulting solution was either dialyzed against 20 volumes of the same buffer for 14 hours or desalted by gel filtration.

4. Gel filtration

The fractions from above were applied to a column of Sephadex G-200 measuring 2.5 x 45.0 cm. Ascending chromatography was attained using a polystatic pump and a 4-way valve (Pharmacia) to reverse the flow. The sample, in 4 ml of buffer, was applied through a Luer valve and eluted with 700 ml of the same buffer.

PART IV: ELECTROPHORESIS ON POLYACRYLAMIDE GEL

In an effort to identify the naturally-occurring substrate of salivary gland protein kinase, a few studies of the phosphorylated protein were tried by separating it and known proteins by electrophoresis. A modification of the discontinuous gel and buffer system of Ornstein (1964) and Davis (1964) was used in which 0.5% agarose is incorporated into the polyacrylamide gel in order to increase the resolution for higher molecular weight compounds. The step-by-step description of the method, using a Canalco gel electrophoresis apparatus, model 12, was followed according to Sierens (1969). 100 µl of phosphorylated homogenates of parotid and submandibular were layered on top of the stacking gels and 50 µl of tracking dye. Electrophoresis was

started at a constant current of 2 mA per tube at 4°C. When the tracking dye had migrated 5 cm into the separating gel, the current was turned off, the gels removed and the protein bands visualized by staining with Coomassie Blue dye. Gels were sliced with a gel slicer and the slices dissolved in 30% hydrogen peroxide at 50°C for 2 hours. The solubilized gels were dispersed in 0.5 ml of NCS (Nuclear Chicago Solubilizer) and the radioactivity counted in Toluene-PPO as described under Methods I-D.

EXPERIMENTAL RESULTS

PART I: PROTEIN CONTENT OF SALIVARY GLANDS

A. BEFORE ISOPROTERENOL

As discussed under Methods (I-B-4), the criterion used to judge the uniformity of the subcellular fractions was their protein content, for many reasons. The acceptable distribution in parotid of normal, starved rats was an average of 10% of the total protein in each of the particulate fractions and 70% in the soluble fraction. Typical values per gland (wet weight 170±5 mg) obtained from the pooled parotid glands of 4 rats (140±4 g) are shown in the first column of Table III. This is a purely arbitrary fractionation, as explained earlier, and was obtained by using consistent methods of centrifugation, homogenization, washing, pH adjustment, etc. It was much more difficult to obtain this distribution of protein in submandibular fractions because of a tendency for agglutination to occur.

B. AFTER ISOPROTERENOL

Following an intraperitoneal injection of 3.3 mg of dℓ-isoproterenol per 100 g wet weight, the wet weight per gland drops to 136±10 mg by the end of 1 hour after injection. Most of this change is due to the excretion of salivary fluids but very significant changes result from the excretion of salivary

TABLE III

PROTEIN CONTENT OF RAT PAROTID GLAND FRACTIONS FOLLOWING ISOPROTERENOL-INDUCED SECRETION

	Control rats (normal, starved) mg	Following isoproterenol treatment 10 min mg	30 min mg	1 hour mg	2 hours mg	4 hours mg	6 hours mg
Homogenate	16.2±1.20	*13.3±1.53	*7.6±0.95	*6.8±1.63	*8.4±1.50	*9.5±1.75	10.8±2.31
Fractions							
Nuclear	1.6±0.13	1.6±0.15	1.5±0.29	1.0±0.25	0.9±0.12	1.3±0.15	1.8±0.24
Mitochondrial	1.9±0.15	1.6±0.18	1.4±0.18	1.6±0.21	1.7±0.24	1.7±0.21	1.8±0.27
Microsomal	1.3±0.10	1.1±0.14	1.0±0.19	0.8±0.13	0.8±0.13	0.9±0.16	1.1±0.21
Soluble	11.2±1.02	8.9±1.10	*4.4±0.95	*3.5±0.56	*3.8±0.56	*4.2±0.56	*4.8±0.59

The protein content per gland was determined in the homogenates and subcellular fractions of the pooled parotid glands of 4 rats (140±4 g) following intraperitoneal injections of 3.3 mg of dl-isoproterenol per 100 g body weight (120 μmoles per kg). The mean values ± S.E.M. are shown for triplicate assays.

*$P < 0.05$

enzymes as well. Gaunce (1971) demonstrated that the parotid gland is secreting α-amylase at a maximal rate by 10 min following isoproterenol treatment. As shown in Table III, the protein content of parotid gland has dropped significantly ($P<0.05$) within 10 min. The distribution of protein in the fractions changes but most of the decrease occurs in the soluble fraction which contains the contents of the lysed zymogen granules. At 1 hour, the α-amylase is over 90% excreted and the protein content of the homogenate is at a minimum. Resynthesis of the protein is apparent between 2 and 6 hours although both the protein content and the wet weight of the gland are well below control values at the end of 6 hours. The decrease in submandibular gland protein concentration after isoproterenol is much less significant because it does not synthesize α-amylase and its excretable enzymes are not produced in such quantity.

Specific activity is the usual manner of expressing the activity of an enzyme but in the parotid gland it becomes apparent that expressing enzyme activity in units per mg protein will lead to erroneous conclusions because the specific activity of any enzyme which is not excreted will more than double as the protein content drops. Nevertheless, reports expressing enzyme activity in rodent salivary glands in this way, appear frequently. It is preferable to calculate enzyme activity in α-amylase-excreting glands as units per gland or per g of tissue, when activity is measured within hours after isoproterenol, in order to partially avoid this artifact.

PART II: CATECHOL O-METHYL TRANSFERASE

A. PROPERTIES

This enzyme has been studied in this laboratory for many years (Pellerin & D'Iorio, 1958; D'Iorio & Leduc, 1960; D'Iorio & Mavrides, 1962; Anderson & D'Iorio, 1968). It has been purified 200-fold and its properties, at least in liver, thoroughly studied. In salivary glands it is found to behave in a linear fashion for 40 min (Figure 11). It also shows linearity of activity with amount of enzyme present in the assay (Figure 12), well beyond the 25 µl of homogenate used in these assays (Methods II-A). It is inhibited by concentrations of SAM above 60 µM as described by McCaman (1965).

B. SUBCELLULAR DISTRIBUTION

The homogenate of parotid glands of normal, starved rats was found to transfer 27.7 (Table IV) to 29 (Table V) nmoles of $^{14}CH_3$ per 175 mg gland per 30 min. This is similar to the 0.304 µmoles found to be transferred per g of brain tissue (wet wt.) by McCaman (1965) per hr. Submandibular gland was found to contain approximately double this amount of activity (Table IV). The homogenates from the pooled glands of 4 normal, starved rats weighing 140±4 g were fractionated, as described under Methods (I-B), and the COMT activity assayed in triplicate. The results, shown in Table IV, indicate that between 40 and 60% of the COMT activity of salivary glands is located in the particulate fractions. In 1965, Alberici et al. also reported

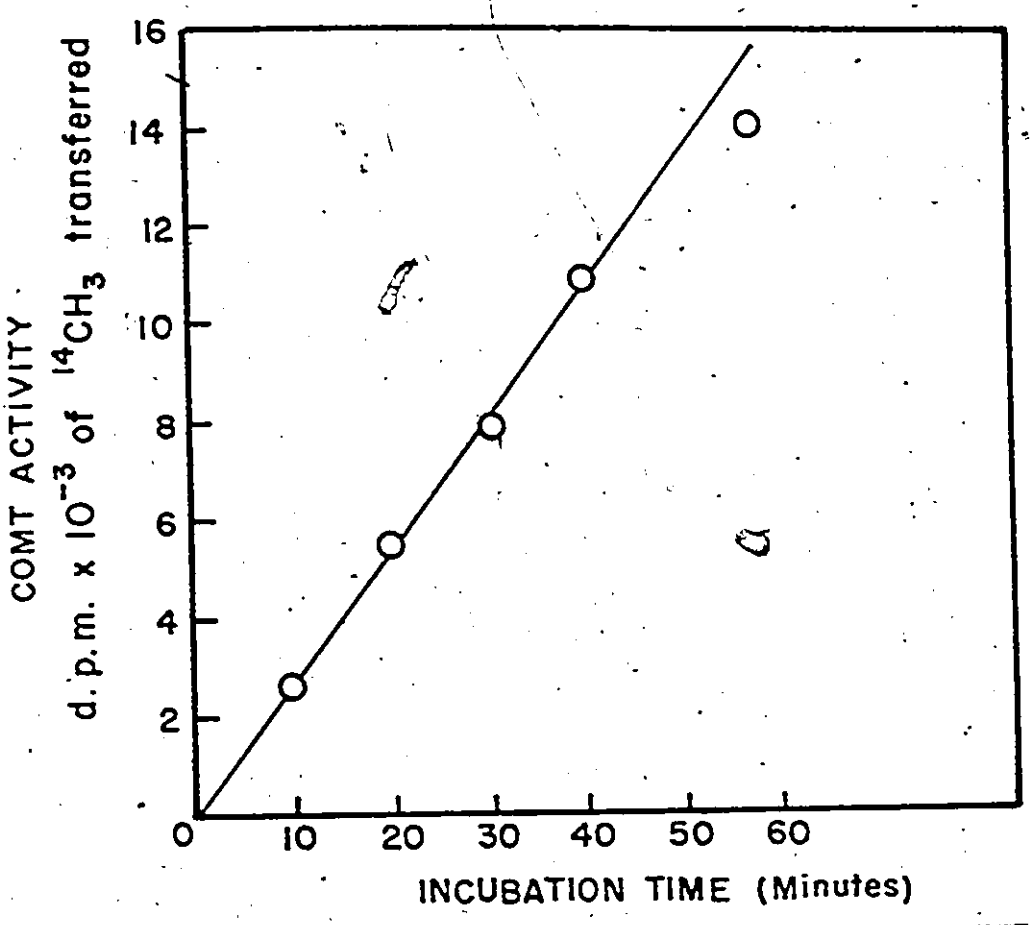


FIGURE 11. LINEARITY OF SUBMANDIBULAR COMT ACTIVITY WITH TIME. Activity is expressed as d.p.m. of $^{14}CH_3$ transferred to 3,4-dihydroxybenzoic acid by 25 µl of homogenate.

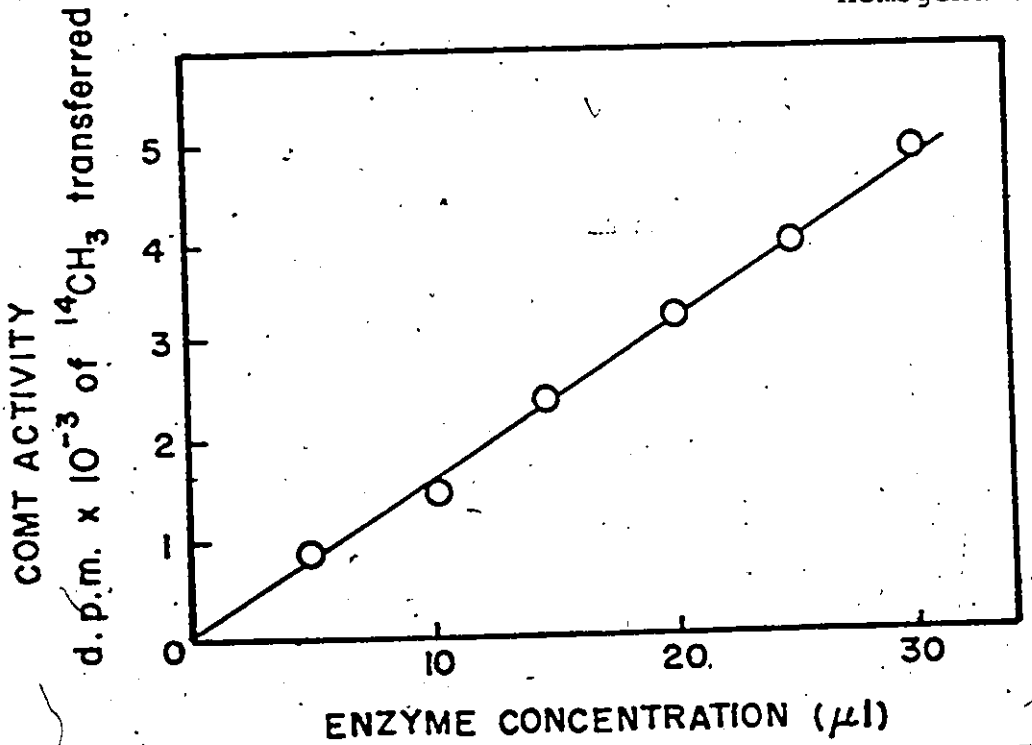


FIGURE 12. LINEARITY OF PAROTID COMT ACTIVITY WITH ENZYME CONCENTRATION (in µl of parotid homogenate added to incubation medium).

TABLE IV

SUBCELLULAR DISTRIBUTION OF CATECHOL O-METHYL TRANSFERASE ACTIVITY IN SALIVARY GLANDS OF NORMAL STARVED RATS

	PAROTID		SUBMANDIBULAR	
	nmoles of $^{14}CH_3$ transferred per gland per 30 min			
HOMOGENATE	27.7 ± 1.73		53.7 ± 2.97	
FRACTIONS:		Per cent distribution		Per cent distribution
NUCLEAR	3.5	16.1	12.1	23.1
MITOCHONDRIAL	4.7	21.5	6.0	11.5
MICROSOMAL	4.7	21.6	4.6	8.8
SOLUBLE	8.9	40.8	29.7	56.7

The mean values of triplicate assays on pooled glands of 4 rats (140±4 g) are shown. The weight per normal parotid gland averaged 175 mg and per normal submandibular gland the mean weight was 164 mg. The homogenates were fractionated as described under Methods (Part I-B).

46% of the COMT activity in rat brain to be located in the particulate fractions.

C. EFFECTS OF ISOPROTERENOL

Fourteen rats, weighing from 130 to 140 g were fasted for 17 hours: 12 were injected i.p. with 5 mg of dl-isoproterenol per 150 g body weight in 1 ml of 0.9% NaCl. The other 2 received 1 ml of 0.9% NaCl only. At intervals of 10 min, 30 min, 1, 2, 3 and 6 hours, the parotid and submandibular glands were removed and homogenized. The COMT activity was determined (Methods II-A) in triplicate in all homogenates. The above procedure was repeated in whole or in part, at least 4 to 6 times for each time interval, and the isoproterenol-induced changes are shown in Figure 13. Both glands show a 3- to 4-fold increase in activity by the end of 1 hour. The changes are parallel in the two glands. The increase in COMT activity of submandibular gland is significant by 10 min ($P<0.005$) and that of parotid gland is significant within 30 min ($P<0.01$). Gaunce (1971) noted an increase in COMT activity at 4 hours after isoproterenol: this is confirmed (Figure 13), as COMT activity does not return to control levels until approx. 6 hours after isoproterenol treatment.

D. EFFECT OF PUROMYCIN

To test whether the increase observed in COMT activity is the result of de novo protein synthesis, 6 rats ranging in weight

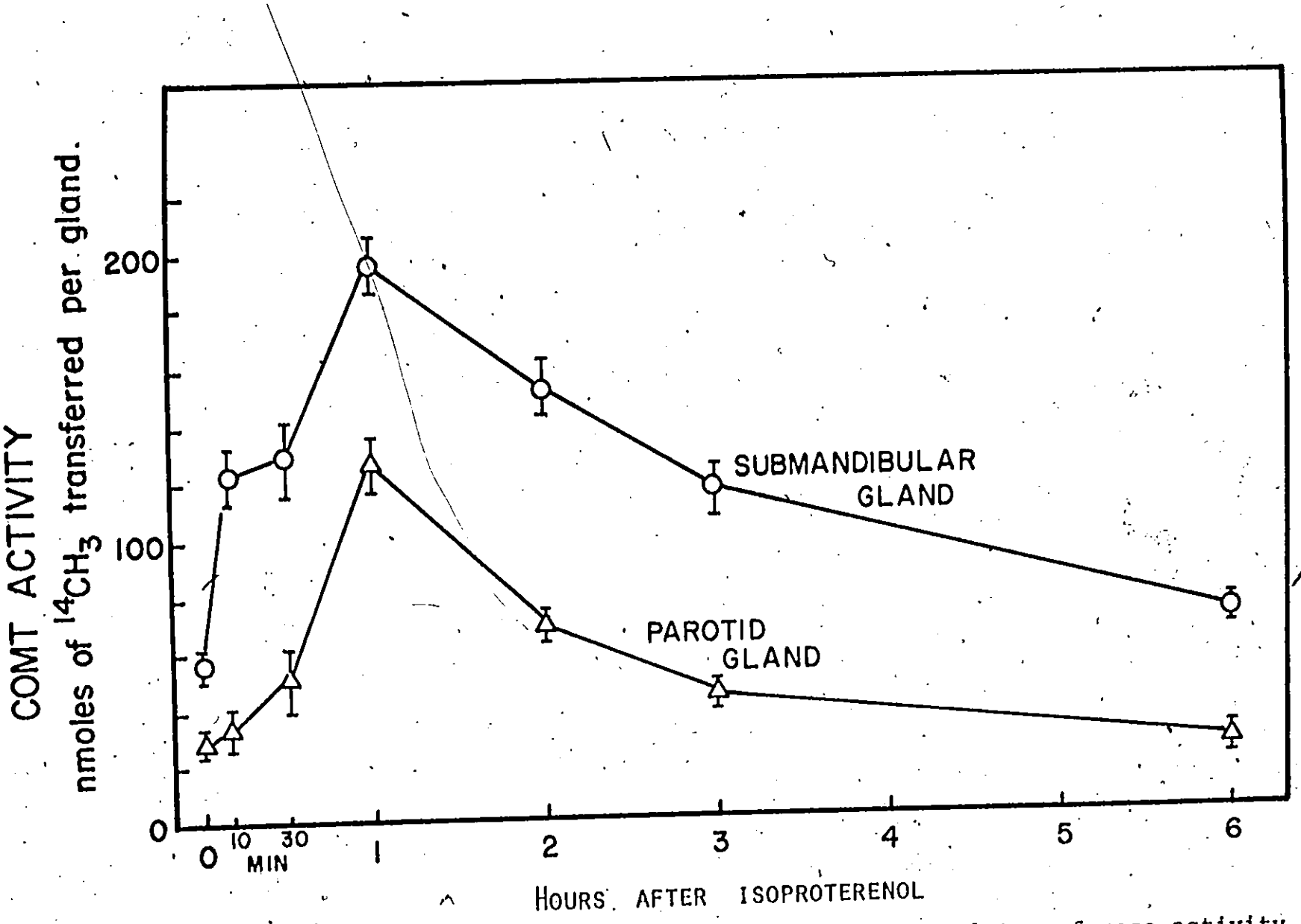


Figure 13. Isoproterenol-induced changes in catechol O-methyl transferase activity in salivary glands. Each point represents the mean ± S.E.M. of 8 rats in each group. Animals were administered 3.3 mg of dl-isoproterenol per 100 g body weight.

TABLE V

EFFECT OF PUROMYCIN ON ISOPROTERENOL-INDUCED CHANGES IN RAT SALIVARY GLAND COMT ACTIVITY

	Normal, starved rat (injected with isotonic saline)	Isoproterenol treated (2 hours)	Isoproterenol plus puromycin (2 hours)
	nmoles of $^{14}CH_3$ transferred per gland per 30 min.		
PAROTID GLAND	29.03 ± 2.54	*68.66 ± 4.65	*75.37 ± 0.20
SUBMANDIBULAR GLAND	58.54 ± 1.85	*152.95 ± 10.18	*321.29 ± 2.18

Pooled glands of 2 rats were used in each group and the mean values ± S.E.M. for 3 determinations are shown in each case. Isoproterenol treated rats received 3.3 mg of isoproterenol per 100 g body weight and those treated with puromycin received the same dose of isoproterenol plus 10 mg of puromycin in two doses spaced 1 hour apart. Enzyme activity is expressed as nmoles of [$^{14}CH_3$] transferred from [$^{14}CH_3$]-methyl-SAM to 3,4,-dihydroxybenzoic acid under standard assay conditions described under Methods (II-A). $p<0.01$)

from 130 to 140 g were treated as follows: 2 received 5 mg of dl-isoproterenol per 150 g body weight plus 10 mg of puromycin (in two doses); 2 received 5 mg per 150 g body weight only and the other 2 received 1 ml of 0.9% NaCl. At 2 hours after the first injection, the parotid and submandibular glands were removed and the COMT activity determined as described in Methods (II-A). Table V shows COMT activity in parotid and submandibular glands after isoproterenol and puromycin. It is apparent that puromycin does not inhibit the synthesis of the enzyme at the translational level, at least, not in the amount received in this experiment. In fact puromycin appeared to stimulate COMT activity, especially in the submandibular gland where the isoproterenol-induced activation more than doubled. Possibly the effect of higher doses of puromycin on isoproterenol-induced changes in COMT activity should be repeated at shorter intervals although there is no evidence that such rapid induction of COMT activity is the result of de novo synthesis of the enzyme as it is in the case of other salivary gland enzymes to be discussed below.

PART III: ADENYLATE CYCLASE

Adenyl cyclase activity is measured by the rate of formation of the product, cyclic AMP. When this project was begun in 1969, many investigators were using some form of chromatography to separate cyclic AMP, e.g. paper chromatography (Pauk & Reddy, 1967); paper electrophoresis (Rabinowitz et al.,1965); column

chromatography (Jungas, 1966; Rodbell, 1967); etc. All of these chromatographic methods were compared by Randerath and Randerath (1964) who showed thin layer, ion-exchange chromatography to be the fastest and most sensitive technique for separation of nucleotides. Although no mention was made of cyclic nucleotides, Randerath (1966) recommended the separation of all other nucleotides on thin layers of unmodified cellulose, treated with poly (ethyleneimine) which has a molecular weight of 30,000 to 40,000. The high resolving power of this impregnated anion-exchange material he attributed to high capacity, great density of functional groups along the PEI chain, and lack of cross linkage, resulting in a high rate of the ion-exchange process. Therefore, it appeared to be the answer to a rapid assay for cyclic AMP, if it could be adapted. After much trial and error in adjusting the elution procedure, etc., cyclic AMP could be separated easily from mixtures of pure nucleotides and bases, even those possessing the same charge. A typical chromatogram is shown in Figure 9 (p.76).

Adapting the method further, for the measurement of cyclic AMP in tissue extracts produced certain difficulties. For example, tissue electrolytes tended to interfere in anion-exchange and to cause streaking of sample nucleotides. After testing many solvents, a 5 min wash in anhydrous methanol was found to overcome the problem, with 90% recovery of tritiated cyclic AMP.

A. ADVANTAGES OF THE METHOD

It was by far the most rapid chromatographic technique; between 12 and 18 samples could be chromatographed on one sheet (20 x 20 cm) and development of each sheet took less than 30 min. Several sheets could be developed at one time.

Another advantage was that levels of ATP and 5'AMP were being assayed at the same time. This was very convenient when it came to testing inhibitors of phosphodiesterase and phosphorolytic enzymes. Also, limitations of other methods could be easily spotted. For example, many methods included a precipitation step, using $ZnSO_4$ and $Ba(OH)_2$ in order to remove ATP, ADP, AMP and inorganic phosphate. It was quite apparent on PEI-cellulose sheets that over 70% of the cyclic AMP was also being removed. Typical c.p.m. found in 10 µl of sample,

before Ba-$ZnSO_4$	and	after Ba-$ZnSO_4$
485		112
398		125
487		129
390		114

indicating that cyclic AMP, with its negative charge, was being precipitated out of the solution as well. This observation could have resulted from too low a pH or incomplete removal of the electroplytes in TLC but even so, methods incorporating this step required corrections for recovery of cyclic AMP of approx. 50% (Bar & Hechter, 1969).

B. PROBLEMS IN ASSAYING ADENYL CYCLASE

1. Low levels of activity

When testing hormonal stimulation of adenyl cyclase in particulate fractions, low levels of activity were encountered by most investigators (Robison et al.,1970) in spite of the fact that cyclic AMP had been discovered originally in a particulate fraction of liver (Sutherland & Rall, 1958). Nevertheless, the amount of adenyl cyclase activity varied directly with the amount of homogenization (Robison et al, 1970) so attempts to measure effects of hormones and other substances on the activity proved frustating. Not only that, the enzyme was extremely heat labile, its activity varied with different batches of enzyme and was progressively lost on standing at 0°C.

2. Multienzyme system

Another major probelm was that the substrate, ATP, is also the substrate for other enzymes present in fifty times the amount of adenyl cyclase, including ATPase and other phosphorolytic enzymes. The enzyme which hydrolyses cyclic AMP to 5'AMP and H^+ was also present. Fortunately these problems could be overcome by the use of phosphodiesterase inhibitors and ATP-regenerating systems.

C. EFFECTS OF VARIOUS AGENTS ON REACTION RATE

In order to measure reaction rates, aliquots of homogenate, or the 200 g particulate fraction, were incubated in 1.0 ml

assays and 0.1 ml aliquots removed at 5 min intervals up to 20 or 30 min. In this way the effects of phosphodiesterase inhibitors, ATP-regenerating systems, various ions, hormones and other agents, on reaction rates, were tested. Because of difficulties in measuring absolute levels, the usual statistical methods could not be applied always. Nevertheless, several characteristics of adenyl cyclase activity were determined.

a) Phosphodiesterase inhibitors: In earliest experiments it became apparent that large amounts of AMP were being formed, even within the first 5 min. As the most obvious cause was breakdown of cyclic AMP by cyclic nucleotide phosphodiesterase, its inhibitors, caffeine and theophylline, were added to the incubation mixture in concentrations up to 30 mM, but the AMP continued to be formed, so other possible causes were investigated.

b) ATP-regenerating systems: It was also apparent that levels of ATP were decreasing significantly within 5 min of starting the incubation (Table VI). When PEP and pyruvate kinase were added to restore ATP concentration, the level of ATP did not decrease so rapidly but was still less than 50% of the starting concentration within 10 min. The addition of NaF helped to maintain the ATP level greatly, possibly by inhibiting some of the phosphorolytic enzymes and the formation of cyclic AMP was linear for at least 10 min under those conditions (Figure 14). When myokinase was added along with PEP-pyruvate kinase, levels of ATP were finally observed to remain stable for 30 min (Table VI). Thus it

TABLE VI

EFFECT OF ATP-REGENERATING SYSTEMS ON ATP LEVELS OF RAT PAROTID GLAND ASSAYS

Incubation period in minutes	No ATP-regenerating system	PEP-pyruvate kinase without NaF	PEP-pyruvate kinase with NaF	PEP-pyruvate kinase, NaF + myokinase
	d.p.m.of ^{14}C-ATP in 10 µl of incubation mixture			
0	14,432	21,646	21,470	22,680
5	6,015	16,237	20,632	23,180
10	2,823	8,654	19,191	21,990
15	1,423	9,081	17,155	22,870
20	1,000	8,416		
25	774	4,868	13,330	23,450
30	813	4,136		24,130

The above changes in [ATP]were observed when 0.1 ml aliquots were removed from a 1.0 ml assay containing 3 mM ATP. The aliquots were placed in a b.w.b.* for 2 min, centrifuged and 10 µl samples chromatographed on PEI-cellulose thin layers , along with an ATP standard solution. The spots were visualized under u.v., cut out and counted in toluene-PPO scintillator fluid in a Liquid Scintillation Counter.

*boiling water bath

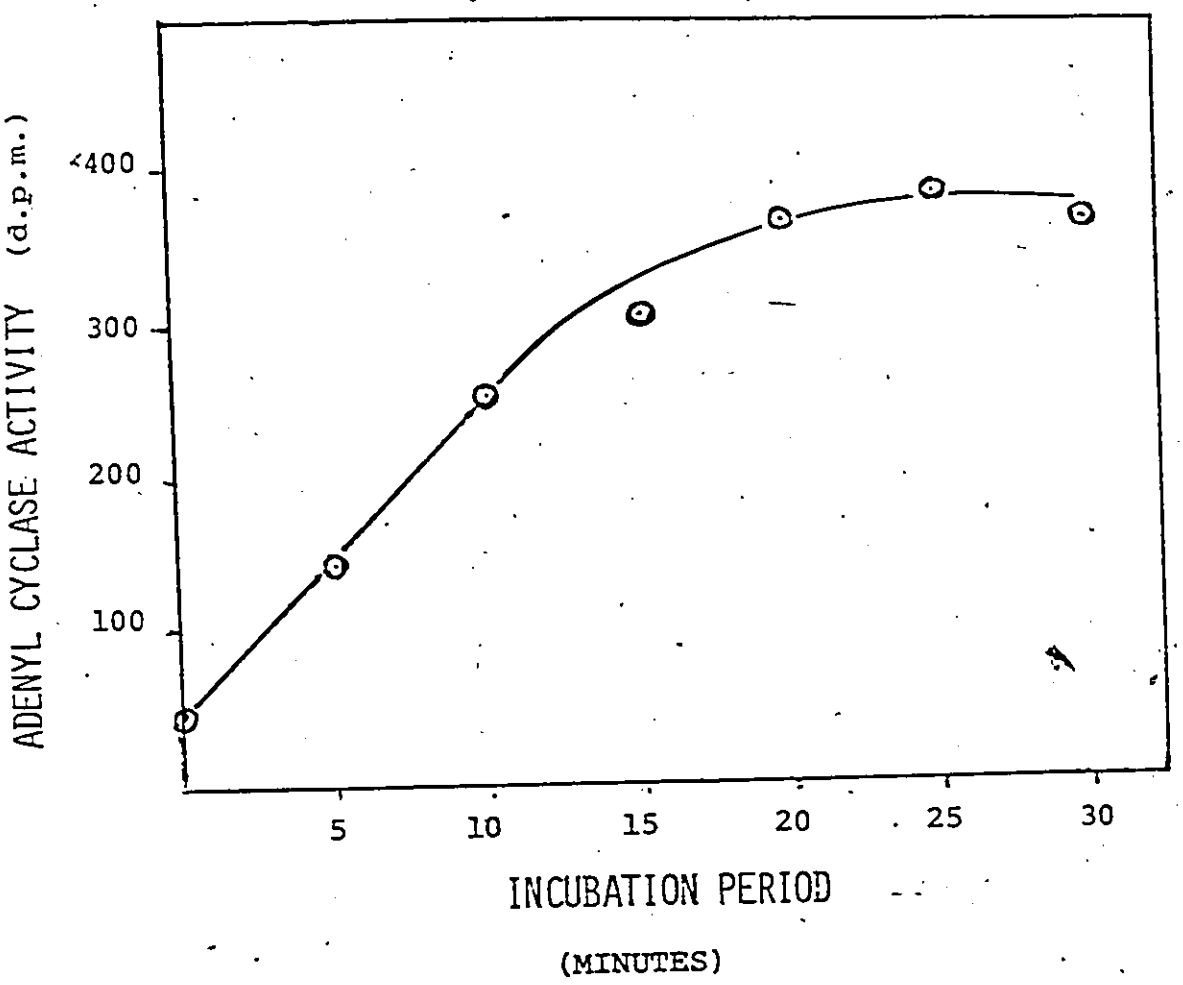


FIGURE 14. LINEARITY WITH TIME.

From a standard 1.0 ml cyclase assay, aliquots of 100µl were removed at timed intervals and used for analysis by the TLC method, applying 10 µl of sample to sheets of polyethyleneimine cellulose. Even though an ATP-regenerating system was included (PEP + pyruvate kinase) the reaction is linear in parotid homogenate for only 10 minutes.

was apparent that maintaining the ATP concentration was a most important factor in salivary glands. When the apparent K_M for ATP was determined in the presence of NaF and the PEP-pyruvate kinase ATP-regenerating system only, it was higher (Table VII and Figure 15) than in later experiments in which the ATP concentration was maintained by the addition of myokinase to the assay and the apparent K_M was lowered to 5×10^{-4}. Bar and Hechter (1969) reported that they obtained a lower K_M for ATP of fat cell ghosts by using [α-^{32}P]-ATP in order to avoid interference from radioactive side products, such as hypoxanthine, which is formed from uniformly-labelled ^{14}C-ATP. To test whether similar interference was occurring in our samples, [α-^{32}P]-ATP was substituted for ^{14}C-ATP and several assays repeated. No difference in adenyl cyclase activity was observed so long as the concentration of ATP was maintained.

In calculating the amount of cyclic AMP formed, Bar and Hechter (1969) suggested that, when separating cyclic AMP by TLC, the per cent conversion of ATP to cyclic AMP be calculated from the d.p.m. in the spots of cyclic AMP and ATP: $\frac{\text{d.p.m. of cyclic AMP}}{\text{d.p.m. of ATP}} \times 100$.

If we re-examine the data in Table VI, it is apparent that, even in the presence of an ATP-regenerating system (such as that used by Bar and Hechter (1969) the concentration of ATP falls rapidly in salivary glands. Thus, calculating by that method gives an unduly large value for per cent incorporation, unless the amount of cyclic AMP also decreases. The reaction has been shown to be reversible (Greengard & Kuo, 1970) but the d.p.m. of cyclic

TABLE VII

REACTION RATES FOR CYCLIZATION OF ATP

BY ADENYL CYCLASE OF RAT PAROTID

[S] (ATP) M	$\frac{1}{[S]}$	v = observed velocity (nmoles of c-AMP formed per mg protein per 10 min)	$\frac{1}{v}$
4.5×10^{-3}	2.22×10^{2}	0.10	10.0
3.5	2.86	0.09	10.9
3.0	3.33	0.085	11.8
2.0	5.00	0.067	15.0
1.5	6.66	0.055	18.2

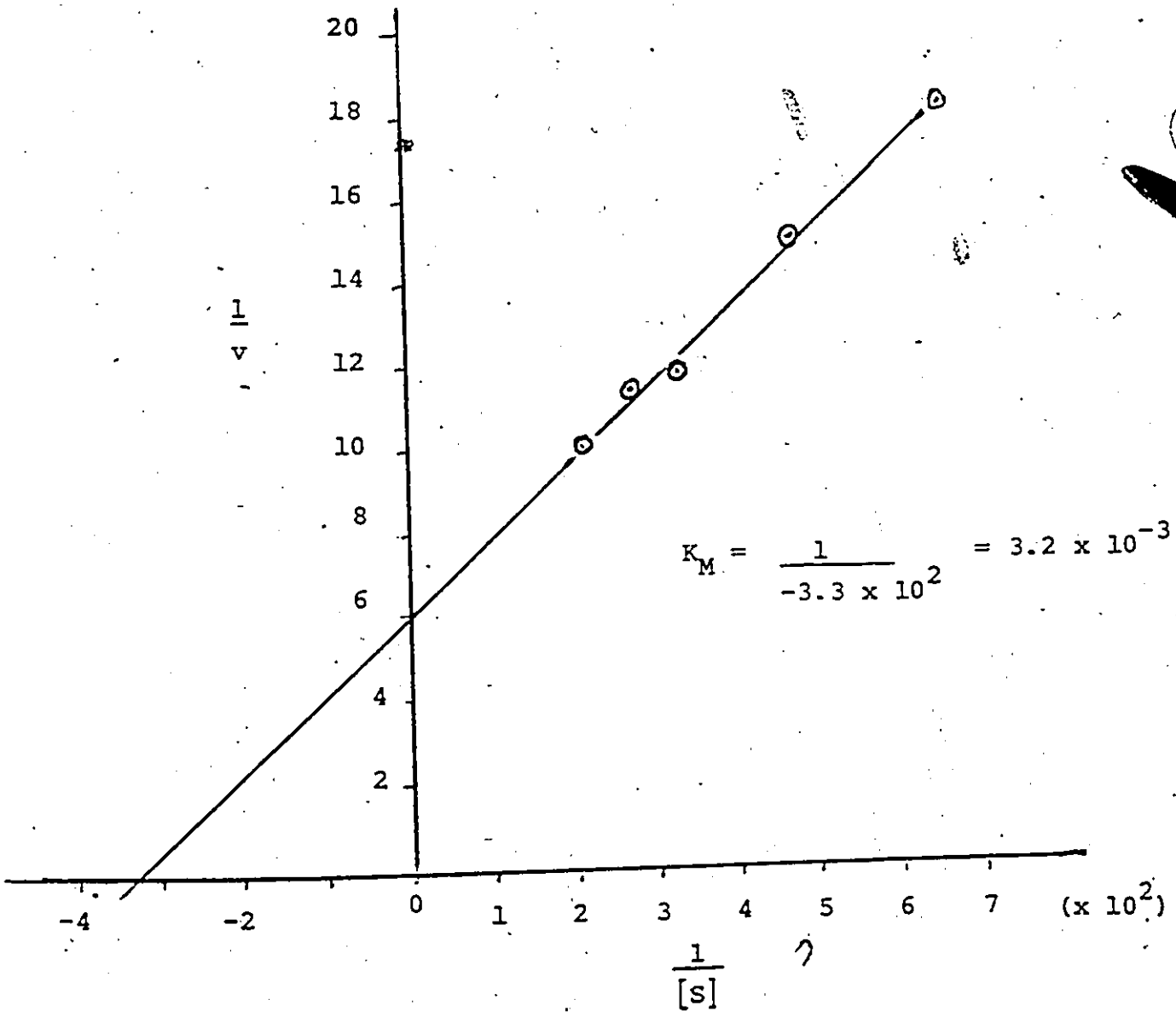


Fig. 15. Apparent K_M for ATP of rat parotid adenyl cyclase assayed in the presence of an ATP-regenerating system and Na fluoride.

AMP were never found to decrease when ATP levels declined, indicating that a pyrophosphatase was also at work to rapidly remove the other product, pyrophosphate, necessary to reverse the reaction. Basing the per cent incorporation of d.p.m. of [^{14}C]-ATP or [α-^{32}P]-ATP present at 0 time was a consistent method but including myokinase, in later assays, was a better solution.

c) Effect of added catecholamines: In spite of the difficulties arising from homogenizing the tissue, time required to dissect parotid gland, interference from other enzymes, etc. by repeating the experiments many times, enough evidence was accumulated to show that isoproterenol and noradrenaline both produce some activation of adenyl cyclase activity in salivary gland homogenates in vitro. In Table VIII it is apparent that the addition of either of these catecholamines (50 µM) produced significant increases in adenyl cyclase activity in both parotid and submandibular glands within 30 min in all cases and much sooner in most cases. The addition of isoproterenol to parotid gland homogenate increased the activity significantly within 5 min. Significant changes with addition of either catecholamine appeared sooner in parotid gland homogenate than submandibular gland homogenate. Adenyl cyclase activity was lower in submandibular gland but other factors, such as the amount of homogenization, could account for that observation.

d) Sodium fluoride effect: Incubation of parotid gland homogenate with no additions to the standard assay (Methods, II-B-2a) produces only small changes in adenyl cyclase activity (Table VIII).

TABLE VIII

EFFECT OF CATECHOLAMINES ON ADENYL CYCLASE ACTIVITY IN RAT SALIVARY GLAND HOMOGENATES

Incubation time	PAROTID			SUBMANDIBULAR	
	No additions (Controls)	Noradrenaline 50 µM	Isoproterenol 50 µM	Noradrenaline 50 µM	Isoproterenol 50 µM
min	picomoles of [^{14}C]-ATP transformed per mg protein				
0	0.38 ±0.01	0.50 ±0.04	1.0 ±0.53	0.53 ±0.06	0.90 ±0.07
5	0.37 ±0.13	2.33 ±0.13	*7.2 ±0.96	1.55 ±0.14	1.93 ±0.26
10	0.49 ±0.17	2.56 ±0.04	8.4 ±0.78	1.05 ±0.23	2.08 ±0.37
15	1.53 ±0.30	*3.86 ±1.03	*13.7 ±1.24	2.81 ±0.11	2.26 ±0.25
20	1.87 ±0.21	*3.85 ±0.05	*14.4 ±0.98	*3.47 ±0.06	2.79 ±0.95
30	1.93 ±0.34	*7.63 ±0.29	*14.8 ±0.87	*4.74 ±0.64	*3.65 ±0.33

Mean values ± S.E.M. represent adenyl cyclase activity in 10 different salivary gland homogenates with and without added catecholamines. The standard assay included an ATP-regenerating system, but no NaF or myokinase, in 1.0 ml, from which 0.1 ml aliquots were removed at the times specified. Enzyme activity was determined by measuring the formation of [^{14}C]-cyclic AMP by TLC (Methods, II-B-2b). With no additions, changes in activity were identical in both glands so 1 set of control values only is shown.

*$P < 0.05$

Addition of myokinase to the standard assay gave more optimal conditions and the activity , with no additions, was higher in Table IX. However, in the presence of sodium fluoride, there was roughly 4 times as much cyclic AMP formed at the end of 20 min.

e) Effect of calcium ion: The role of Ca^{2+} in salivary gland function has become increasingly important since it was demonstrated to be essential for induction of secretion in parotid gland slices (Selinger & Naim, 1970). An ATP-Ca^{2+} complex is believed to play a key role in determining membrane structure and function (Rasmussen & Tenenhouse, 1968) who suggested that the action of adenyl cyclase converts the cyclic AMP from a strong chelator of Ca^{2+} to a weak chelator, thereby leading to the release of Ca^{2+} and changes in membrane structure conducive to excretion. Calcium uptake by microsomal preparations of rat salivary glands has been reported to be ATP-dependent (Selinger et al., 1970).

Rat salivary glands contain a high concentration of calcium in relation to other soft tissues (Dreisbach, 1957; Feinstein & Schramm, 1970). The formation of cyclic AMP has been reported to be calcium-dependent in some tissues (Bradham et al.,1970; Shimizu et al.,1970) and calcium-independent in other cases (Birnbaumer et al.,1970).

In order to test whether the high endogenous level of Ca^{2+} in salivary glands plays a part in activating adenyl cyclase, the specific Ca^{2+} chelator, EGTA, was used to examine adenyl cyclase activity in the absence of Ca^{2+}. Although the experiments were repeated many times, the relative effects are best demonstrated in the

TABLE IX

EFFECT OF CALCIUM ION ON PAROTID ADENYL CYCLASE ACTIVITY

Time of incubation	No additions	Added NaF (10 mM)	Added EGTA (10 mM)	Added EGTA + NaF	Added EGTA + Ca^{2+} (10 mM)
	Picomoles of cyclic AMP-^{32}P formed per mg protein				
0 minutes	0.8	1.2	8.6	18.5	2.3
5	2.3	7.2	11.0	10.1	17.0
10	3.3	6.0	17.0	19.4	8.9
15	4.5	9.9	20.9	28.1	9.8
20	3.9	17.4	21.5	21.8	12.8

To show the effect of removing the Ca^{2+} present in parotid gland, by chelation, EGTA was added to the standard assay containing an ATP-regenerating system plus myokinase. Samples of parotid nuclear (200 g) fraction were incubated in a 1.0 ml assay and 0.1 ml aliquots were removed at the times specified. The [α-^{32}P] incorporated into cyclic AMP was assayed by TLC, as described under Methods (Part II-B-2b).

comparative study (Table IX) in which all assays were performed at same time. When EGTA was added to the assay, the amount of cyclic AMP formed by 20 min was even greater than that formed by NaF activation. In the absence of Ca^{2+} the activation by NaF was much more rapid, i.e. almost maximal in the time necessary to add the enzyme, mix and remove the first aliquot (0 time). When Ca^{2+} was replaced in the assay, the reaction rate slowed down. Thus there is evidence that removal of Ca^{2+} by EGTA potentiates adenyl cyclase activity in parotid gland. EGTA was also reported to potentiate the adenyl cyclase stimulation by glucagon in liver membranes (Birnbaumer et al., 1970).

f) Basal level of cyclic AMP: In all of the preceding studies of adenyl cyclase activity, the amount of cyclic AMP present at 0 time was measured in order to compare with that produced by activation of the enzyme. As shown in Table VIII and IX, only 0.38 to 0.8 pmoles per mg of protein were found when measuring cyclic AMP by the TLC method on PEI cellulose (Methods, II-B-2b). This was very low compared with values reported by other methods at the time, but recent methods are indeed confirming that basal tissue levels of cyclic AMP are in the range of 0.2 to 1.5 pmoles per mg protein (Steiner et al.,1970; Weller & Rodnight, 1973).

D. EFFECTS OF ISOPROTERENOL ON ADENYL CYCLASE IN VIVO

In this set of experiments, rats weighing 130-140 g were injected intraperitoneally with 33 mg (120 µmoles) per kg body weight. At intervals of 2 min to 4 hours, samples of parotid gland

were removed and processed rapidly (Methods, II-B-2c) to prevent phosphodiesterase activity. The tissue extracts, containing the cyclic AMP formed, were used to activate protein kinase, which had been partially purified (Methods, II-C-5). This partially purified protein kinase was found to be sensitive to nanomolar amount of cyclic AMP. Standard solutions of cyclic AMP were prepared and incubated with 50 µg of enzyme protein. A typical calibration curve is shown in Figure 16. The assay for protein kinase activity is described under Methods (II-C-2). The phosphorylated protein was precipitated and washed until only phosphoester bonds, stable in alkali without heating, were present. Tissue extracts replaced the cyclic AMP in sample tubes and all extracts and standards were assayed in duplicate.

The effect of isoproterenol on adenyl cyclase activity in vivo is shown graphically in Figure 17. The activity is measured as nano moles of cyclic AMP formed per g wet weight because there was not time to dissect out the whole gland under conditions required to preserve cyclic AMP in the tissue. The level of cyclic AMP was found to increase in parotid gland within a few minutes of isoproterenol injection, perhaps within seconds if one could determine it that soon. Within 10 min it reaches a maximum and then rapidly decreases. These data were presented in 1971 and were confirmed, independently, by Guidotti et al., (1972) in mouse parotid. This increase in cyclic AMP tissue level is produced by a dose of catecholamine giving much higher blood levels than those normally found.

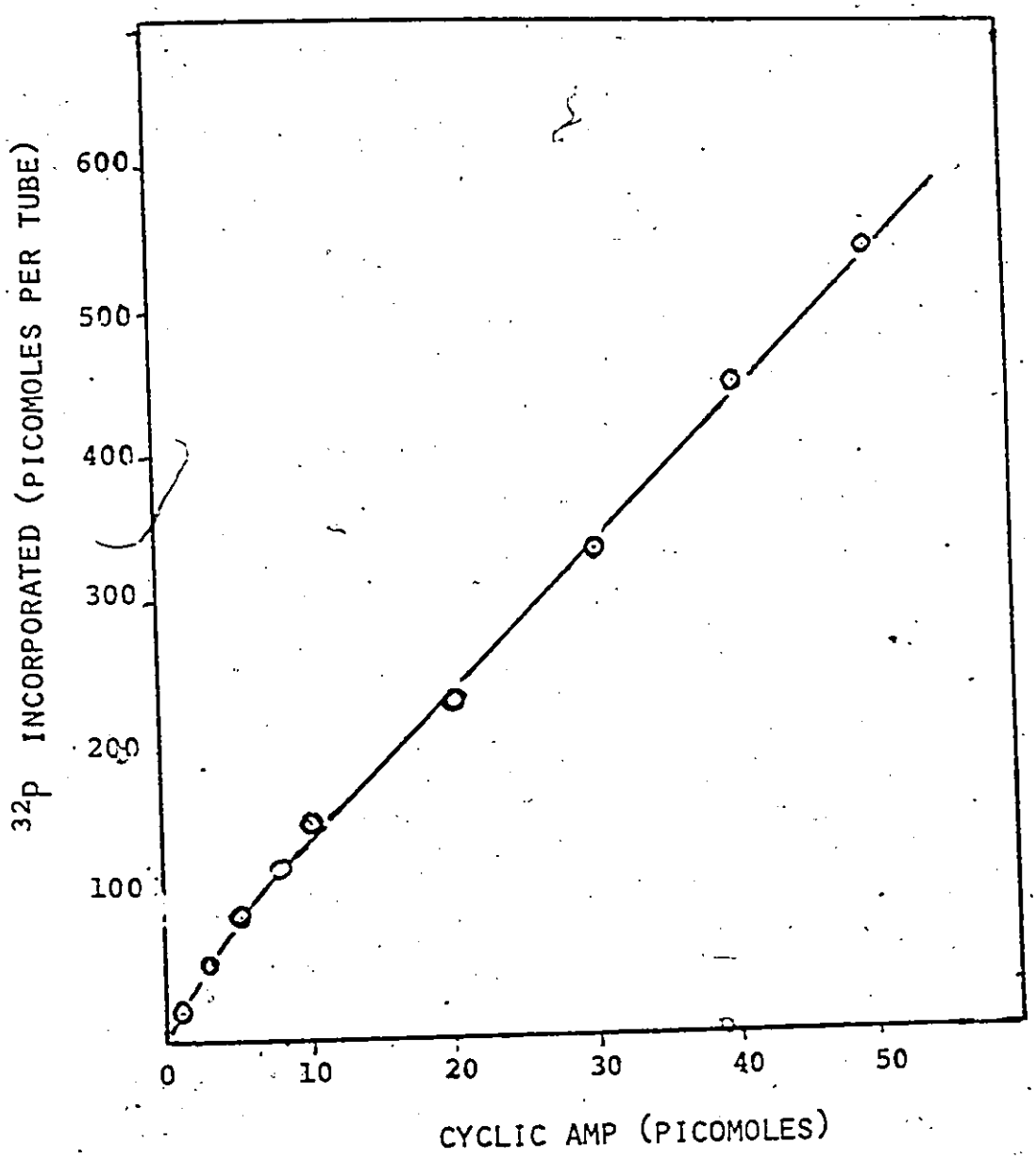


Figure 16. Standard curve for the measurement of cyclic AMP using a partially purified preparation of parotid protein kinase (50µg per tube). The assay volume was 1 ml and 2 mg of histone were added as substrate.

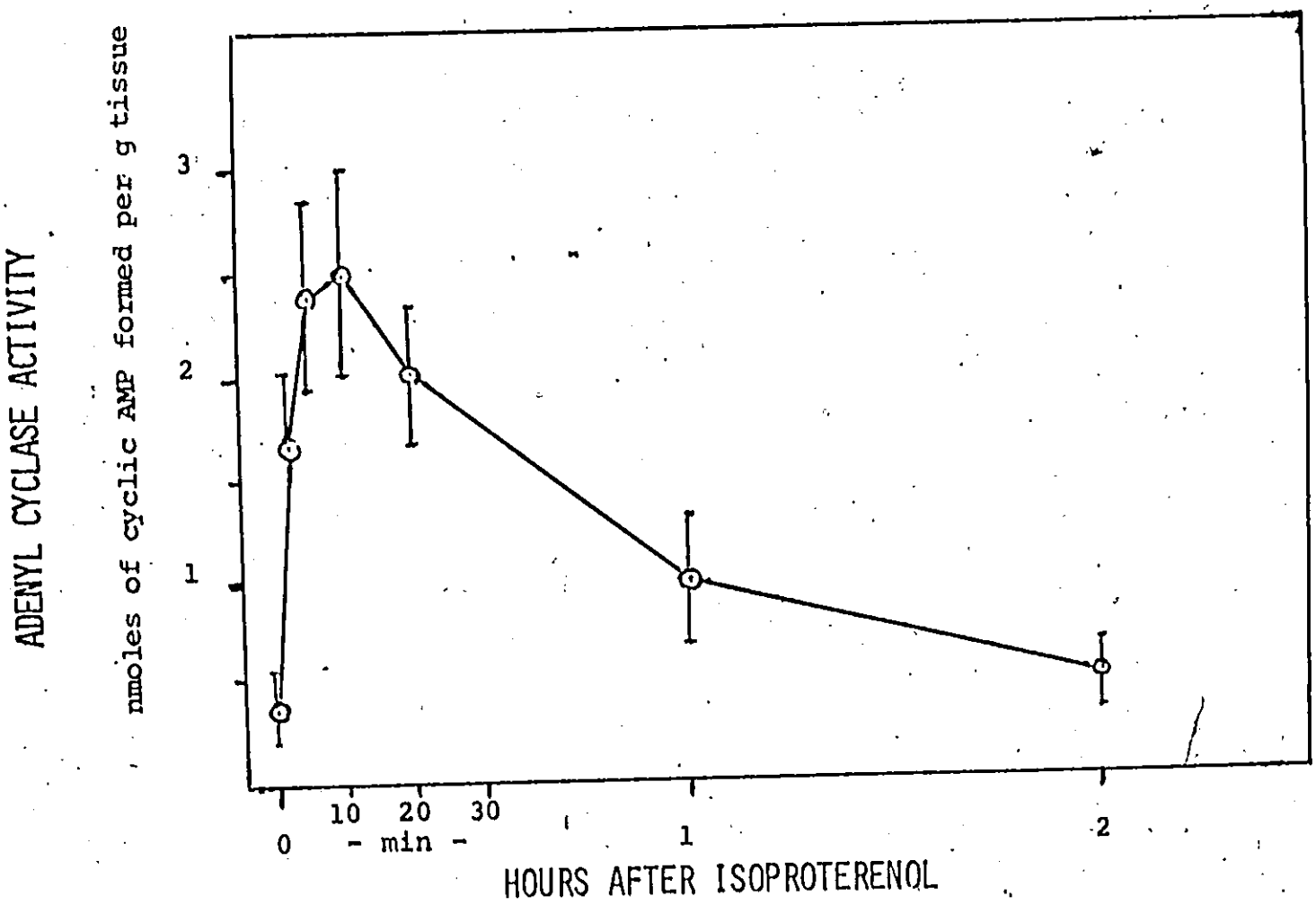


Figure 17. Time course of the isoproterenol-induced stimulation of parotid gland adenyl cyclase: each point represents the mean ± S.E.M. of 4 rats in each group. Animals were injected i.p. with 3.3 mg of dℓ-isoproterenol per 100 g body weight. Cyclic AMP was extracted from parotid glands at intervals shown and used to activate partially purified protein kinase.

PART IV: PROTEIN KINASE

A. CHARACTERIZATION OF THE ENZYME

Under the assay conditions described, the progress of phosphoryl group incorporation into homogenate proteins by endogenous protein kinase was found to be linear for 5 min. The rate of incorporation was only slightly lower at the end of 20 min. The pH optimum is 7.0 but 6.5 was the pH chosen for the assays because of the high phosphoprotein phosphatase activity in salivary glands. Although phosphatase activity is no doubt involved in the turnover equilibrium of phosphorylation *in vivo*, the effects of catecholamines on protein kinase only are included in this study, so the effects of protein phosphatases were avoided by using high [Mg^{2+}] and pH 6.5. As observed in the adenyl cyclase assays (Table VI), ATPase activity is great, also, so 10 mM NaF was included in all assays. Maximum protein kinase activity was demonstrated using 9 to 14 mM Mg acetate. The higher concentration was probably required to overcome the effects of EDTA in the homogenizing medium and EGTA in the assay. Both EDTA and EGTA are necessary to remove the high endogenous [Ca^{2+}] present in salivary glands because Ca^{2+} has been found to inhibit protein kinase activity in most tissues (Kuo *et al.*,1970). The enzyme was found to phosphorylate basic proteins, such as histone much more readily than acidic proteins, such as casein which appears to inhibit phosphorylation of natural substrates (Fig.18).

The protein kinase of parotid gland can be stored for 4 weeks at -15° and remains stable, in fact, its activity is found

to increase on storing, in the same way as protein kinase of cardiac muscle (Brostrom et al.,1970). This indicates that it consists of an inactive (R) and an active (C) subunit. These two subunits become dissociated by oxidation, possibly of -SH groups, to yield the active, cyclic AMP-independent protein kinase. A great deal of the active, cyclic AMP-independent form of the enzyme is present in parotid gland (Table X). It phosphorylates naturally-occurring substrates, with no additional substrates or activators added to the standard assay

As observed by other workers, there are differences in the absolute levels of protein kinase from different rats (Maeno et al.,1971). Because of this fact, combined with the fact that the endogenous activity increases on storing, the results of assays performed on stored samples are skewed and application of the usual statistical methods gives erroneously high deviations; however, the relative changes are still significant, and confirm observations noted in typical studies where up to 150 assays were conducted in one day to overcome the effects of storage.

B. SUBCELLULAR DISTRIBUTION

1. Endogenous activity

The level of endogenous protein kinase activity in pooled parotid glands of normal, fasted rats was investigated first. The glands were homogenized and fractionated (Methods I-B-2 and I-B-3) and the activity calculated as picomoles of ^{32}P incorporated per gland per 5 min. The subcellular distribution is shown. (Table X)

TABLE X

DISTRIBUTION OF PROTEIN KINASE ACTIVITY IN SUBCELLULAR FRACTIONS OF RAT PAROTID GLAND

	No additions	Histone	Histone plus cyclic AMP	Triton X-100	Triton X-100 plus histone
	picomoles of ^{32}P incorporated per gland per 5 min				
HOMOGENATE	1,277 ±221	*3,478 ±685	**7,272 ±493	*2,647 ±504	*3,743 ±702
FRACTIONS:					
Nuclear	75 ± 32	*251 ± 48	**640 ± 90	*287 ± 67	**514 ± 75
Mitochondrial	132 ± 38	290 ± 57	470 ± 85	770 ± 95	864 ± 88
Microsomal	122 ± 29	267 ± 43	382 ± 75	874 ±127	775 ± 82
Soluble	777 ±388	*1,902 ±422	**4,869 ±783	*1,905 ±403	**3,679 ±506
% recovery	80	78	87.5	145	156

The values shown are the mean ± S.E.M. of 4 to 6 different experiments using 4 glands from 2 normal, starved rats each time. Protein kinase activity was assayed within 4 weeks, with and without the above-mentioned additions to the standard assay (Methods, II-C-1). Because the activity increases on storage, the deviations are high but the relative changes are still significant; *$P < 0.02$ and **$P < 0.01$.

Close to 70% of the activity is present in the high speed supernatant; the other 30% is distributed between the particulate fractions. Only 80% of the activity of the crude homogenate is recovered in the fractions. This may reflect displacement of the natural substrate during fractionation or may indicate the presence of an inhibitor, such as that reported to be present in skeletal muscle (Walsh et al, 1971). The endogenous activity of the homogenate varies from 600 to 1400 units per gland, depending on the length of time of storage.

2. Latency of protein kinase activity

The addition of 0.1% Triton X-100 (octyl phenoxy polyethoxyethanol from Sigma Chemical Co.) to the assay causes the release of endogenous activity in all fractions. As shown in Table X, the increases are greatest in the mitochondrial and microsomal fractions. The relative distribution is observed to change. Now 50% of the activity is present in the particulate fractions. Although the activity in the total homogenate is more than double that with no additions, the detergent brings out additional latent activity in the fractions so that 145% of the activity of the homogenate is recovered. Adding histone along with Triton X-100, produces an additive effect in some fractions indicating that the additional activity is produced by different mechanisms. The detergent may dissolve phospholipids in the membrane, exposing new enzyme molecules, or it may even dissociate the inactive holoenzyme by allosteric effects.

C. EFFECTS OF HISTONE AND CYCLIC AMP

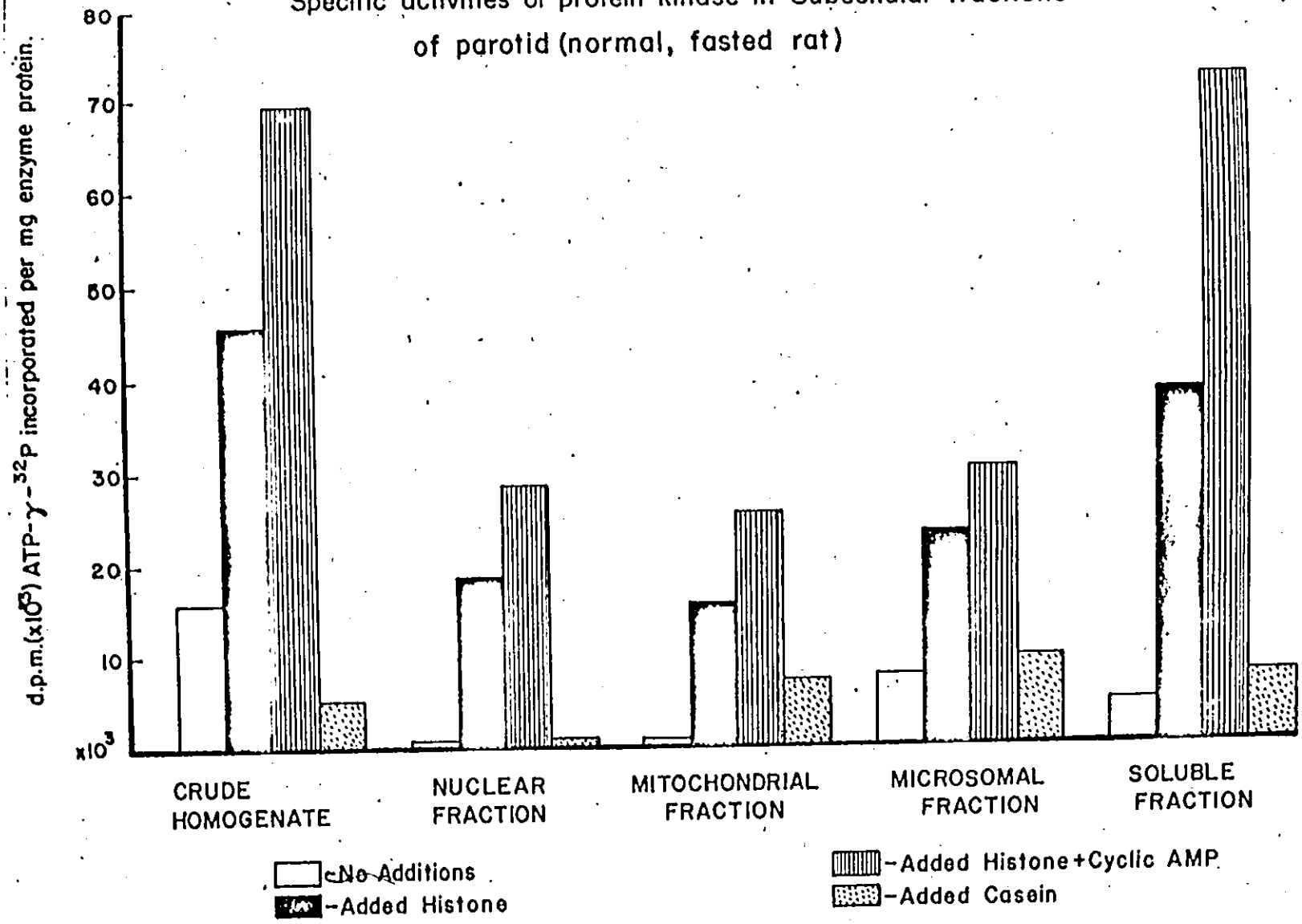
The increases in the specific activity of each fraction in the presence of added histone and cyclic AMP in the parotid glands of normal, fasted rats, is shown in Figure 18. It is apparent that there is much enzyme activity, even in the absence of added cyclic AMP so it cannot be said that the enzyme is completely dependent on cyclic AMP *in vitro*. The soluble fraction shows the greatest specific activity with added histone plus cyclic AMP, although all fractions respond with great increases in specific activities, especially when histone is added first. This may reflect merely the lack of natural substrate (s) displaced during differential centrifugation or it may be due to the activating effect of basic proteins (Tao, 1972; Miyamoto *et al.*,1971). In preferring a basic protein, such as histone, as substrate, this protein kinase is similar to those observed in other tissues, such as skeletal muscle (Walsh *et al.*,1968); liver (Langan, 1968); cardiac muscle (Brostrom *et al.*,1970); frog bladder (Jard & Bastide, 1970); and many other tissues (Kuo *et al.*,1970).

D. EFFECTS OF ISOPROTERENOL TREATMENT

Normal rats, which had been fasted overnight, were injected intraperitoneally with 33 mg (120 μmoles) per kg body weight of dℓ-isoproterenol in 1 ml of isotonic saline. Control rats received 1 ml of isotonic saline only. At the end of 1 hour, the parotid glands from each rat were pooled, fractionated (Methods, I-B-3) and the protein kinase activity of the homogenate and each

FIGURE 18

Specific activities of protein kinase in Subcellular fractions of parotid (normal, fasted rat)



fraction determined according to the standard assay described in Methods II-C-1. The changes in protein kinase activity at 1 hour after isoproterenol injection are shown in Table XI. The intrinsic activity in the homogenate nearly doubles by the end of 1 hour. This increase in activity is reflected in the soluble fraction as well. A significant increase is observed in the nuclear fraction where the mean value for picomoles of ^{32}P transferred to protein nearly triples. The protein kinase activity is expressed as picomoles of ^{32}P transferred per gland (rather than per mg enzyme protein) because the salivary enzyme proteins are excreted so rapidly after an injection of isoproterenol.

Changes in isoproterenol-induced activity which occur between 2 and 6 hours after treatment are shown in Figure 19, which records observations of another typical study. In this study, homogenates were centrifuged only once, at 27,000 g to obtain a soluble fraction which includes microsomal particles and a particulate fraction consisting of unwashed nuclear and mitochondrial particles. Again, increases over control values are found in both soluble and particulate fractions. Note that the number of units of activity per gland is lower at 2 hours than at 1 hour in the total homogenate but appears to increase again at 6 hours. A similar decrease at 2 hours followed by an increase for several hours was observed in rat submandibular gland nuclei by Ishida and Ahmed (1973) following isoproterenol injection. In their study, a much higher dose of isoproterenol was used (160 mg per kg body

TABLE XI

ISOPROTERENOL-INDUCED CHANGES IN PAROTID PROTEIN KINASE ACTIVITY

	CONTROL RATS (normal, starved)	ISOPROTERENOL-INJECTED RATS 1 HOUR
	picomoles of ^{32}P incorporated per gland	
HOMOGENATE	1,370 ± 157	*2,583 ± 236
FRACTIONS:		
NUCLEAR	146 ± 48	*456 ± 67
MITOCHONDRIAL	198 ± 56	299 ± 68
MICROSOMAL	162 ± 45	320 ± 53
SOLUBLE	825 ± 103	*1,725 ± 127

Means ± S.E.M. represent at least 4 animals in each group. Control rats were injected intraperitoneally with 1 ml of isotonic saline and treated rats received 3.3 mg (12 μmoles) per 100 g body weight. At the end of 1 hour each pair of glands was homogenized and fractionated as described under methods (I-B-3) and protein kinase assayed by standard assay conditions for 7 min.

*$p < 0.05$

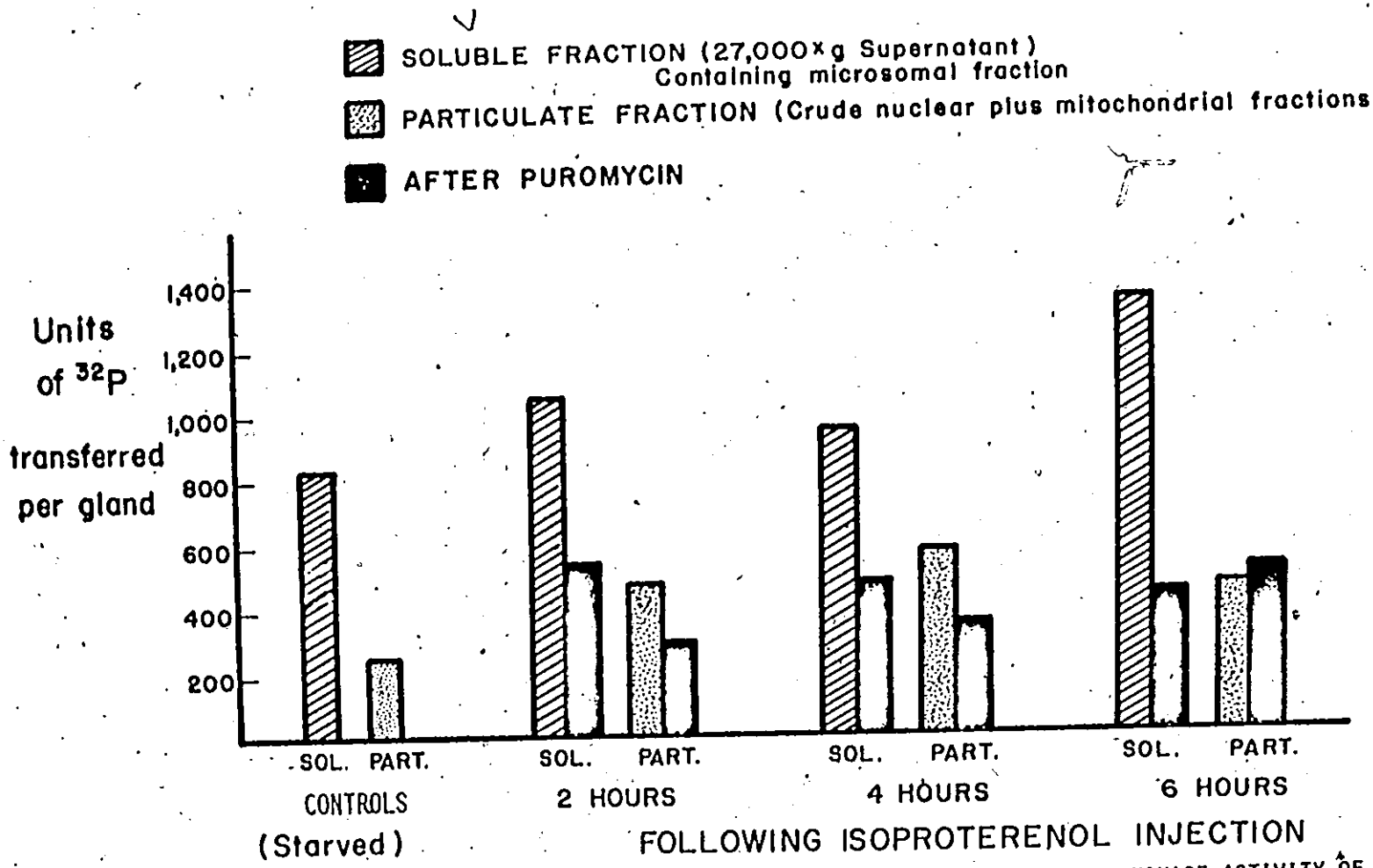


FIGURE 19. THE EFFECT OF PUROMYCIN ON ISOPROTERENOL-STIMULATED INTRINSIC PROTEIN KINASE ACTIVITY OF RAT PAROTID GLAND. EACH BAR REPRESENTS THE MEAN VALUE FOR 4 RATS IN EACH GROUP. THE RATS WERE INJECTED INTRAPERITONEALLY WITH 3.3 MG OF ISOPROTERENOL PER 100 G BODY WEIGHT EXCEPT FOR THE CONTROL GROUP WHICH RECEIVED ISOTONIC SALINE ONLY. IN ADDITION, HALF OF THE ISOPROTERENOL INJECTED RATS RECEIVED 20 MG OF PUROMYCIN PER 100 G IN DIVIDED DOSES. THE GLANDS WERE POOLED AT TIMES INDICATED, THEN FRACTIONATED AND PROTEIN KINASE ASSAYED, IN TRIPLICATE, IN SOLUBLE AND PARTICULATE FRACTIONS.

weight) and measurements were made only at 2, 16 and 24 hours after injection.

E. EFFECTS OF PROTEIN SYNTHESIS INHIBITORS

To test whether any of the observed increases in protein kinase activity depended on new protein synthesis at either the transcriptional or translational levels, protein synthesis inhibitors were injected, at the same time as isoproterenol, and the effects on isoproterenol-induced increases observed. Because the enzyme and its substrate are both protein in nature, the effect of a protein synthesis inhibitor could involve either the enzyme, its regulatory subunit or its substrate.

1. Puromycin

Figure 19 shows the effect on protein kinase activity, or on the amount of protein phosphorylated, when 200 mg per kg body weight is injected intraperitoneally at the same time as isoproterenol. Because puromycin is very toxic, the dose must be given in several injections at hourly intervals. Unfortunately the effect on protein kinase activity and amount of protein phosphorylated at 1 hour after isoproterenol was not tested, but as shown in Figure 19, there is much less ^{32}P transferred from [γ-^{32}P]-ATP to endogenous proteins at 2, 4 and 6 hours after isoproterenol, especially in the soluble fraction (containing microsomal fraction). It is of interest that the same dose of puromycin completely prevented the incorporation of ^{14}C-amino acids into salivary gland

proteins and prevented the resynthesis of α-amylase (Gaunce,1971). Actinomycin D has no inhibitory effect on α-amylase resynthesis, so Grand and Gross (1970) suggested that the synthesis of this enzyme, and possibly other salivary gland proteins, as well, are controlled at the translational level.

2. Actinomycin D .

The effect of actinomycin D on isoproterenol-induced increases in protein phosphorylation was tested at 4 hours only. Four rats were injected with 1 mg per kg body weight of actinomycin D dissolved in ethanol and diluted to 1 ml with isotonic saline. Control rats were injected with 1 ml of isotonic saline only. Eight rats received intraperitoneal injection of 5 mg of dl-isoproterenol per 150 g body weight and 4 of these rats were injected with 1 mg per kg of actinomycin D as well. The results are shown in Figure 20. There was no significant difference between control rats and those injected with actinomycin D, nor was there any significant difference in protein kinase activity of parotid homogenates between groups injected with isoproterenol or isoproterenol plus actinomycin D. Thus, actinomycin D had no effect on protein kinase activity in either control or treated rats. However, the increase in protein kinase activity, measured as ^{32}P-phosphorylated proteins, was very significant ($P<0.01$) in all isoproterenol-treated rats. As outlined in the Review (II-B-4c), protein synthesis inhibited by actinomycin D, soon after isoproterenol injection, has been related to DNA synthesis (Barka,1965b; Baserga & Heffler,1967).

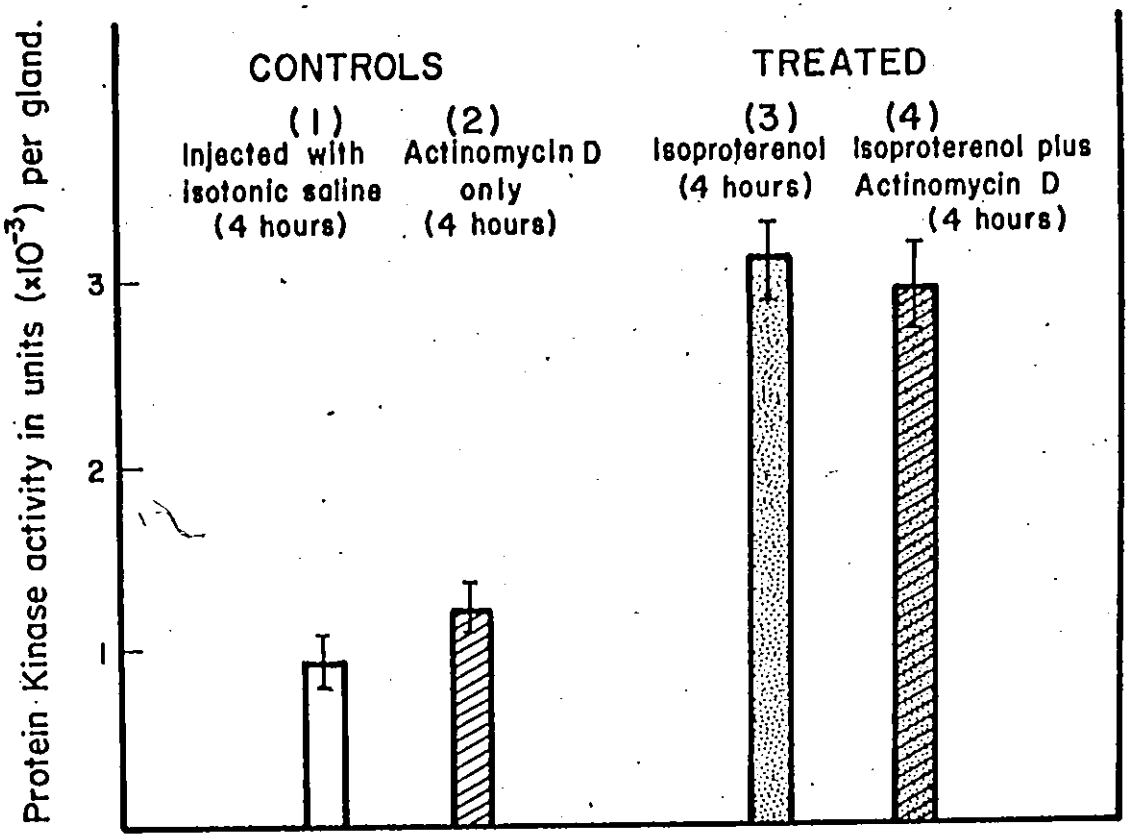


FIGURE 20. Actinomycin-insensitive stimulation of endogenous parotid protein Kinase Activity after Isoproterenol. Four rats were used for each determination. The means and the S.E.M. are shown.

F. PARTIAL PURIFICATION OF PROTEIN KINASE

1. Increase in specific activity

The 27,000 g supernatant was submitted to ion-exchange chromatography, salt fractionation and gel filtration, as described under Methods, Part III. The increase in specific activity observed at each purification step is shown in Table XII. By performing the five steps the specific activity increased roughly 10-fold. The greatest increase in specific activity occurred when the enzyme protein was subjected to column chromatography on DEAE cellulose, so for all practical purposes, such as the assay of cyclic AMP levels (Methods, II-B-2c) the enzyme separated at this step was employed.

2. Characteristics of the partially purified enzyme

A typical elution profile of the proteins eluted after column chromatography on DEAE cellulose, is shown in Figure 21. The tissue used was parotid gland from normal, fasted rats. The main protein peaks are labelled A, B, C, etc.

Peak A contained the greatest amount of endogenous protein kinase activity. It was cyclic AMP-dependent and would readily phosphorylate histone. Except for the increase in specific activity, it was identical with the non-purified protein kinase. It could be further purified by gel filtration.

Peak B contained no protein kinase activity.

TABLE XII

PROTEIN KINASE ACTIVITY IN PAROTID GLANDS AFTER SUCCESSIVE PURIFICATION STEPS

Fraction	Protein mg per ml	Specific activity (nmoles of ^{32}P incorporated per mg enzyme protein)	Yield (%)
Crude homogenate (in 3 volumes of medium, w/v)	27.2	0.220	100
Supernatant 27,000 g	16.7	0.288	80
DEAE cellulose (fraction I)	4.6	1.000	75
$(NH_4)_2SO_4$ precipitate	3.2	1.200	60
Gel filtration	0.4	2.400	40

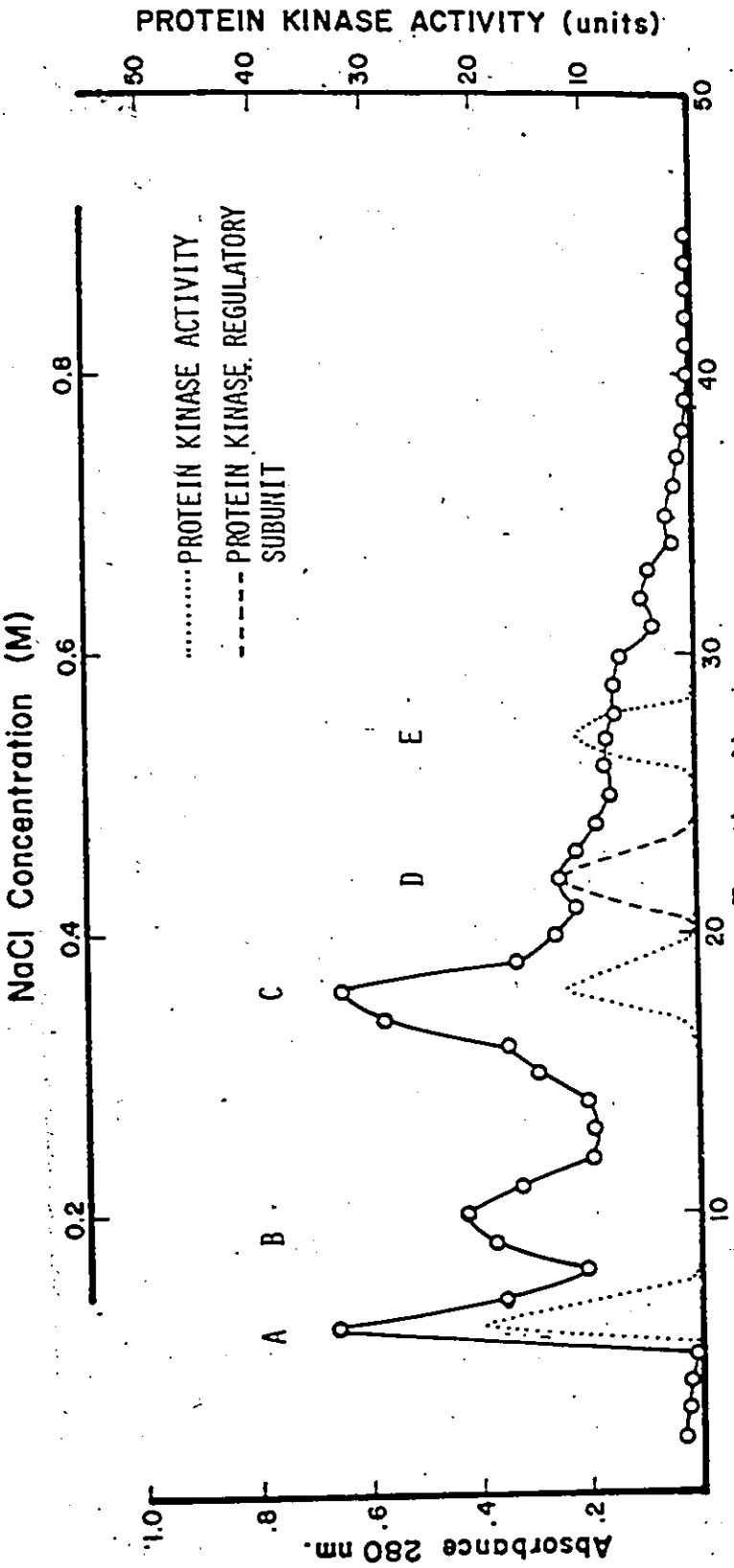


FIGURE 21. PROTEIN KINASE ACTIVITY IN THE SOLUBLE (27,000 g) FRACTION OF RAT PAROTID GLAND SEPARATED BY COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE.

Peak C contained an active protein kinase. It would phosphorylate histone equally well in the absence of cyclic AMP, so was considered to be cyclic AMP-independent.

Peak D contained no protein kinase activity.

Peak E contained protein kinase, similar to that of peak C except that it had a pH optimum of 7.2 instead of 6.8. It could phosphorylate casein as well as histone at that pH.

3. Inactivation of active fractions

In order to determine if the mechanism of activation of parotid protein kinase was similar to that of other tissues, as outlined in the Review (I-D-3), the proteins of the inactive fractions, obtained above, were tested for any effects on the active enzymes of peaks C and E. The protein of peak D was found to completely inactivate the cyclic AMP-independent protein kinases in peaks C and E. To prove that this effect was due to the recombination of the regulatory subunit (R) with the catalytic subunit (C), the combined peaks were chromatographed again on DEAE cellulose. The protein was eluted, this time, in an earlier fraction, similar to peak A in being less strongly adsorbed to the column. When tested for protein kinase activity, with no histone as substrate, this protein was completely inactive unless cyclic AMP was added to the assay. It was thus concluded that protein kinase of parotid gland contains the (R) and (C) subunits which are dissociated by cyclic AMP in the same way as that from many other tissues.

G. SEPARATION OF PHOSPHORYLATED PROTEINS BY ELECTOPHORESIS

1. Natural substrates

To study the nature of the natural substrates for protein kinase, phosphorylated proteins from protein kinase assays of parotid homogenate were subjected to electrophoresis on polyacrylamide gel (Methods IV). The gels were stained with Coomassie Blue to visualize the protein bands and a typical gel, with no addition to the assay, is shown in Figure 22, tube IV. The gel was sliced using a gel slicer (Canalco) and the individual slices were dissolved in 30% hydrogen peroxide (which bleaches the dye). The ^{32}P incorporated into the protein was counted, as described under Methods I-D. The distribution of natural substrates is shown in Figure 22 (bottom). The main substrate in normal homogenate separates at slices 14 to 16. There is another natural substrate at slice 21. Therefore, two, and possibly more, naturally phosphorylated substrates are shown to be present in parotid homogenate.

2. Histone as substrate

With added histone (tubes I to III, Figure 22) an extra, dark band appears between slices 10 to 13. This area is shown to be phosphorylated (broken line) as well as the naturally occurring substrates. It is apparent that one of the natural substrates has an isoelectric point very close to that of histone, type II, but it is not identical.

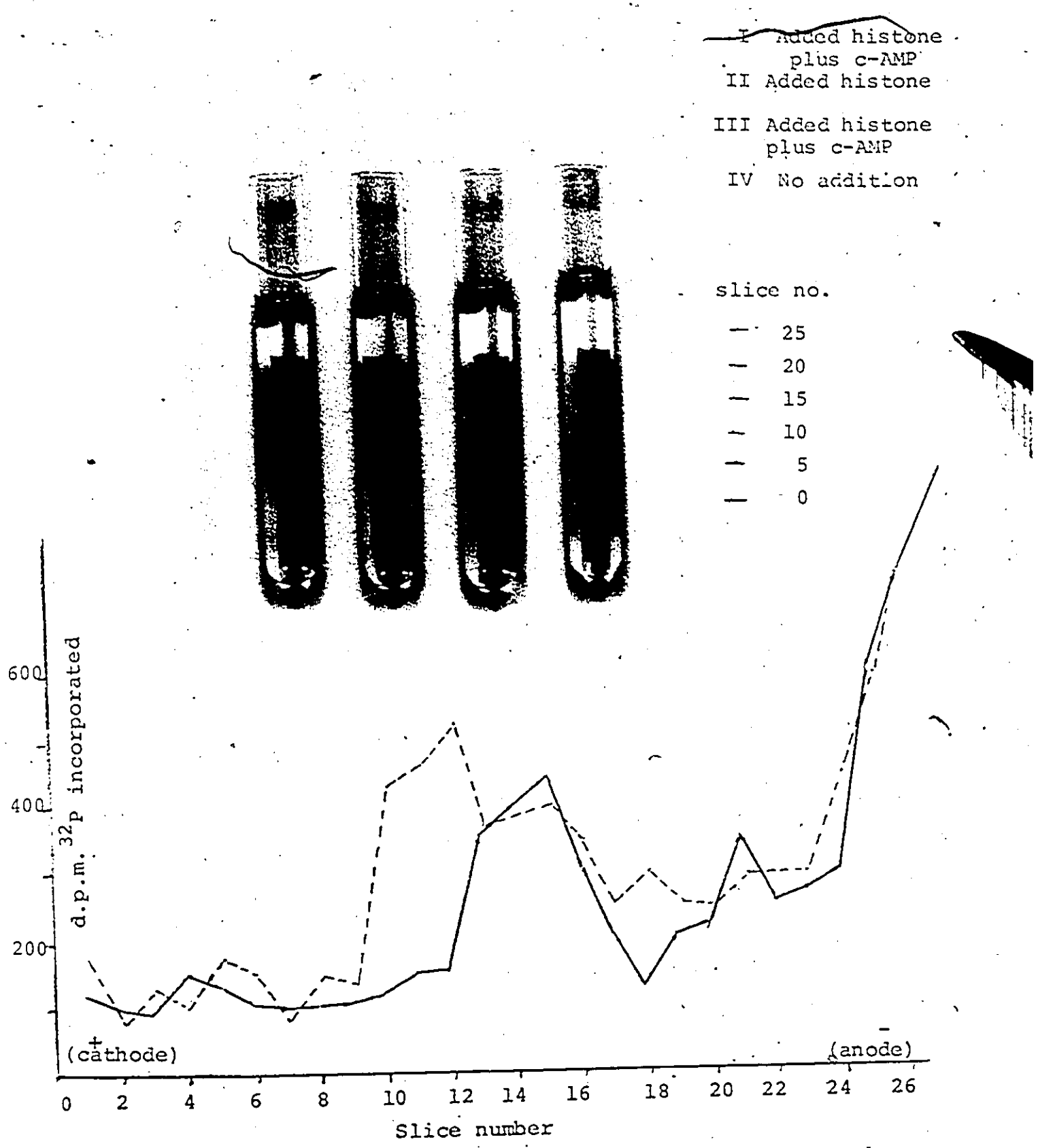


Figure 22. Separation of phosphorylated protein by electrophoresis on polyacrylamide gel. ------- added histone
_____ natural substrates

DISCUSSION

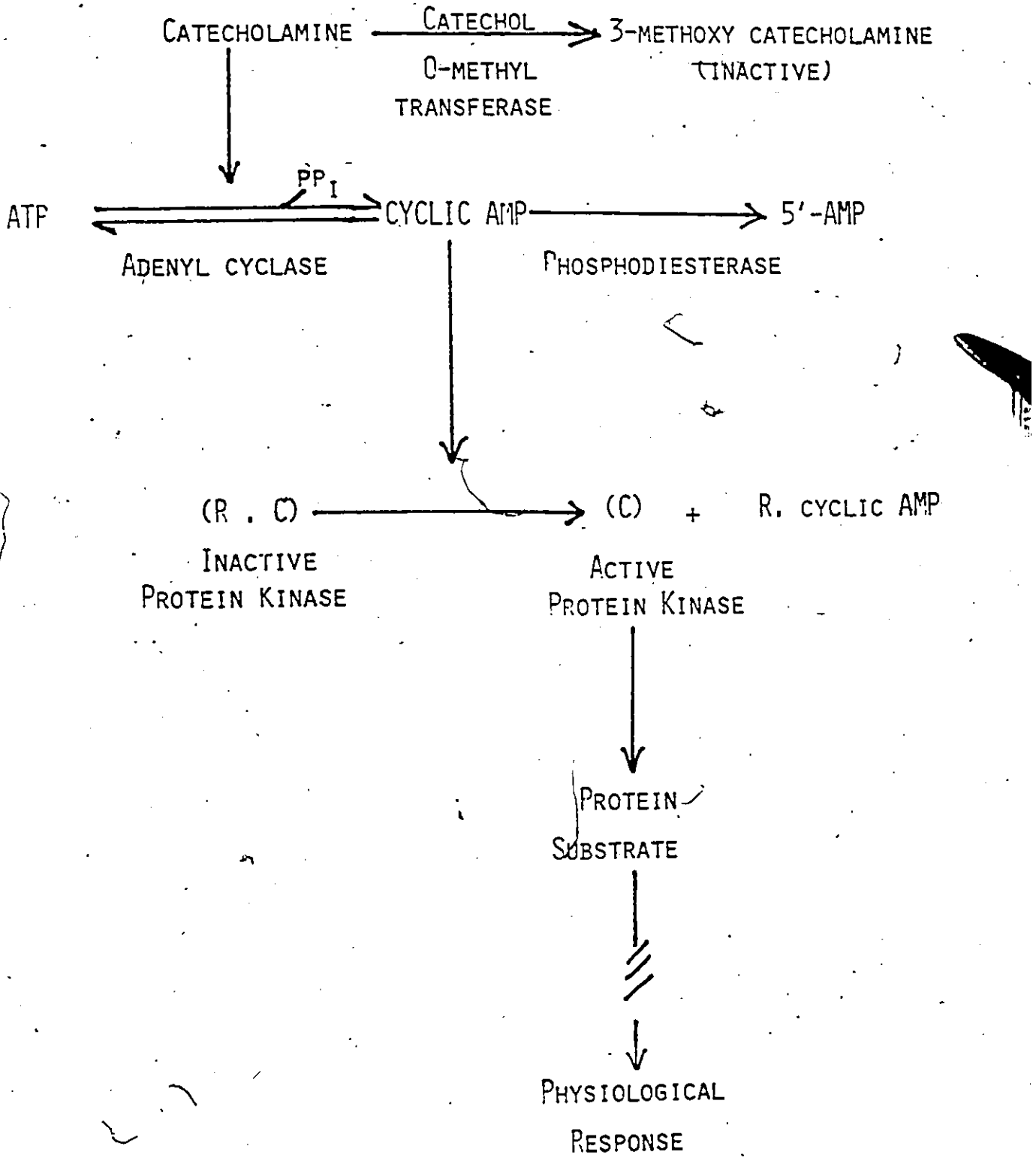
PART I: GENERAL

All of the studies included herein have involved the catecholamine, isoproterenol, and its effect on three enzymes found in salivary glands (i.e. parotid and submandibular glands). As shown in Figure 23, two of the enzymes are closely related to the formation of cyclic AMP and the third is dependent on its formation for activation. A fourth enzyme, phosphodiesterase, is concerned with its inactivation, but has been found to have very low, if any, activity in salivary glands (Weiss et al.,1972).

1. Adenyl cyclase

It is difficult to prove that adenyl cyclase activity is high, or even present in most tissues. There are two reasons for this. In the first place, adenyl cyclase activity is destroyed in broken cell preparations. As shown in Table VIII, only a few picomoles of cyclic AMP are formed per mg of protein and one must repeat the assay many times to achieve conditions under which even small amounts of activation can be proven. Secondly, the basal tissue level of cyclic AMP in salivary glands (i.e. assayed at 0 time, Table VIII) was found to vary from 0.38 to 1.0 picomoles per mg protein only. At the time, this was believed to be due to inefficiency of the method, but on reconsideration, it may be the normal situation. Even if adenyl cyclase is extremely active, there is little reason for high tissue levels to

FIGURE 23. THE ADENYL CYCLASE SYSTEM



exist in normally functioning tissue. This is apparent from examining Figure 23. If cyclic AMP, as it is formed by adenyl cyclase activity, is acting as a second messenger and furthering a biochemical sequence, then it is soon complexed to its acceptor protein, the inactive protein kinase, and is not present in its free form long enough to be measured. If the adenyl cyclase is not being stimulated by a hormone, such as a biogenic amine (catecholamine), then the turnover of cyclic AMP may be low and any cyclic AMP released from the regulatory subunit (R), (sometimes referred to as a reservoir of cyclic AMP) would be hydrolysed by phosphodiesterase. Similarly, if the adenyl cyclase is being activated by a catecholamine, only the turnover rate would increase. Therefore, under no conditions can one picture pools of cyclic AMP waiting to be measured under physiological conditions. It is now confirmed by Weller and Rodnight (1973) and Steiner et al (1970) that basal levels of cyclic AMP range from 0.2 to 1.5 picomoles per mg of protein. Thus, the method used herein (TLC on PEI cellulose) must have been more accurate than supposed at the time.

If the above theory is true, then how does one explain the increase in tissue level of cyclic AMP following isoproterenol treatment of rats, found in salivary glands, by us (Horwood et al, 1971) and by others (Malamud, 1969, 1972; Guidotti et al, 1972)? This may be a non-physiological response, resulting from β-adrenergic stimulation by a very potent catecholamine

in a unique type of gland. Reasons for this suggestion will be discussed at length later in this discussion, in relation to a proposed control mechanism, involving COMT.

2. Catechol O-methyl transferase

Parotid gland of normal, fasted rats was found to contain enough COMT activity to transfer 29 nmoles of [$^{14}CH_3$] per gland per 30 min assay. Each gland averages 175 mg (approx.) so the COMT activity per g wet weight, per hour is 331 nmoles. McCaman (1965) using the same method quotes 304 nmoles per g wet wt per hour as the COMT activity in rat brain with a coefficient of variation of less than 5%. The activity in submandibular gland was more than double that of parotid gland. The COMT activity in monkey submandibular gland was higher than in any other tissue except liver (Axelrod et al., 1959). It seems reasonable to suppose that no enzyme would be present with relatively high activity unless it were there to serve some useful purpose in vivo. In Figure 23, its relations to cyclic AMP levels is apparent: its main function is to deactivate circulating catecholamines before they have a chance to stimulate adenyl cyclase, so in this sense, COMT may play a role in controlling the level of cyclic AMP formed.

3. Protein kinase

Protein kinase in parotid gland was found to be present in an active, cyclic AMP-independent form and also in an inactive, cyclic AMP-dependent form. The addition of a protein subunit,

separated by DEAE cellulose was found to reinstate the active form (Results IV-F-3). The inactive form could be activated by cyclic AMP, so the protein kinase of salivary glands is believed to consist of a regulatory and a catalytic subunit which can be dissociated by cyclic AMP, which binds to the regulatory subunit and causes the release of the catalytic subunit as shown in Figure 23. This is the same mechanism for activation as found in many other tissues (Tao et al.,1970; Gill & Garren, 1971; Erlichman et al.,1971; Yamamura et al.,1971; Kumon et al.,1970; etc.).

Assuming a constant amount of the holoenzyme (R.C) in the tissue, an increase in cyclic AMP in the tissue will shift the equilibrium to the right and a decrease in cyclic AMP concentration will shift it to the left. Hormones have been reported to change the ratio of (C) to (R.C) in just that way (Soderling et al.,1973). The total amount of protein kinase activity in a tissue can be determined by assaying the homogenate in the presence of cyclic AMP and histone and measuring the amount of phosphorylated protein. The activity of the intrinsic protein kinase (C) found in parotid gland (Table X) averaged approx. 1,000 units per 5 min per gland and the total stimulated activity was over 7,000 units. Assuming a constant amount of (R.C) in the tissue, the changes produced by isoproterenol are directly proportional to the amount of cyclic AMP formed by the action of the catecholamine. After 1 hour, the number of units per gland was found to increase to 2583 units.

PART II: ISOPROTERENOL-INDUCED CHANGES

A. CYCLIC AMP LEVELS AND COMT

Within the shortest time that it takes for an i.p. injection of isoproterenol to reach the parotid gland and measurement of cyclic AMP to be initiated, there is an increase in the level of cyclic AMP reflecting adenyl cyclase stimulation in the tissue (Figure 17). This change was observed by others, as well, (e.g. Malamud, 1969; 1972; Guidotti et al., 1972). It increases the level of cyclic AMP to about 2.5 nmoles per g of tissue (wet weight) at 10 min. This is relatively high compared to cyclic AMP levels formed in the lung and heart after isoproterenol injection (Guidotti et al.,1972). In the heart the increase appears to be only about 10 picomoles per mg protein.

Let us examine this great increase in cyclic AMP level in the light of other changes which occur in parotid gland following administration of isoproterenol. The relative changes which occur in adenyl cyclase activity and COMT activity (in nanomole units) are shown in Figure 24. The COMT activity is 10-times higher to start with than the peak activity of adenyl cyclase and the increase is only a fraction of that which actually occurs. The increase in COMT activity is truly dramatic and illustrates very clearly why there is such a small proportion of the dose of isoproterenol unmetabolized after 40 min (Guidotti et al.,1972). The increase in ^{3}H-methoxy-isoproterenol found by Baserga et al., (1969) is also explained.

Now, considering that isoproterenol is such a potent β-adrenergic receptor stimulator and the rapid induction of COMT

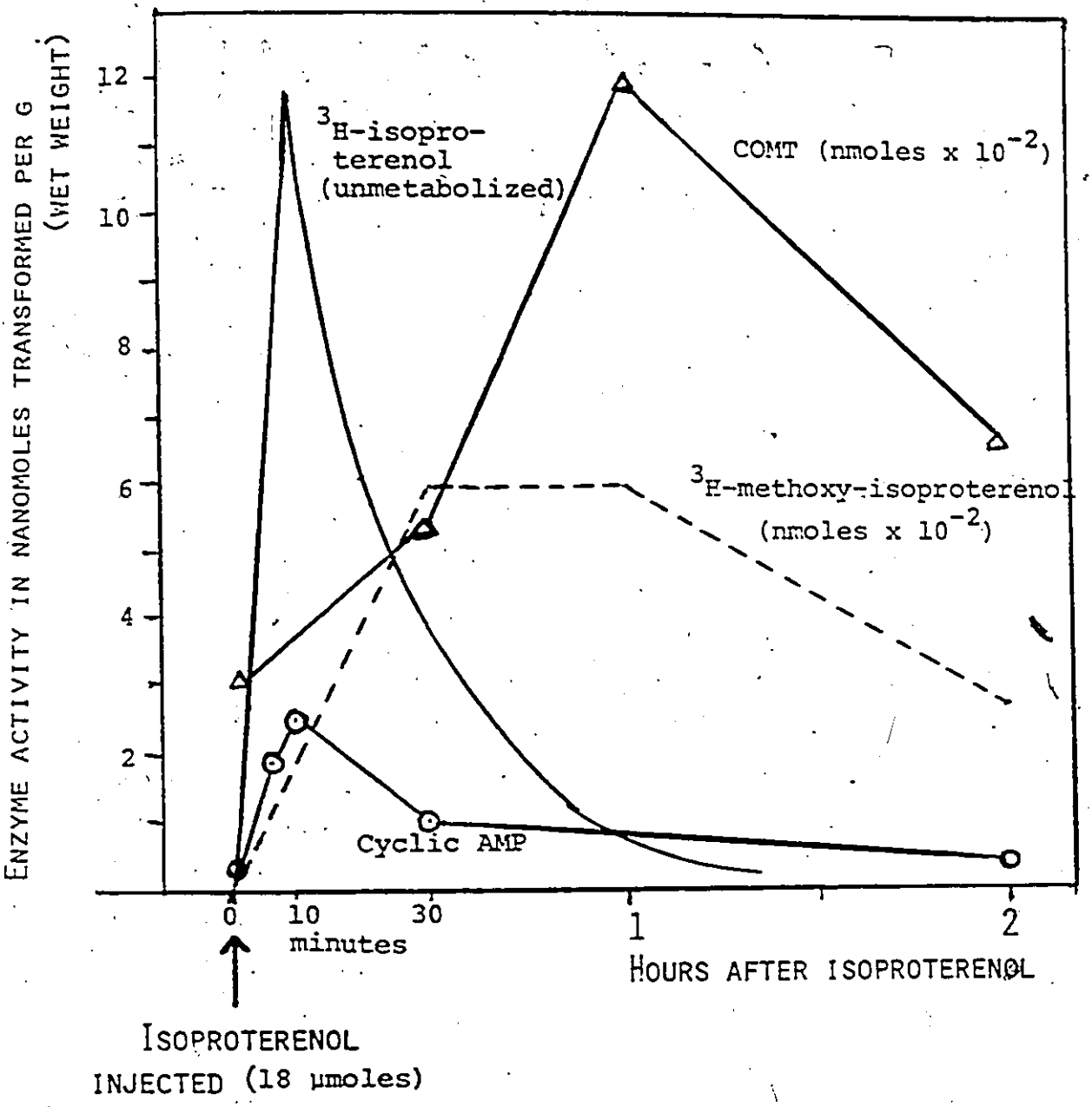


Figure 24. Relative changes in levels of cyclic AMP, COMT activity and 3-methoxy-isoproterenol following an injection of isoproterenol. Rat parotid changes:

———— ^{3}H-isoproterenol (unmetabolized), from Guidotti et al.,1972.

—△—△— COMT activity (from Figure 13)

—○—○— Cyclic AMP level (from Figure 17)

– – – – – – ^{3}H-3-methoxy-isoproterenol levels, from Baserga et al.,1969.

activity to deactive the catecholamine, does it not become apparent that the purpose of the COMT may be to inactivate circulating catecholamines in a tissue which is controlled in vivo by neural reflexes through the autonomic nervous system?

Under no physiological conditions would the parotid gland encounter such a strong β-adrenergic agonist. Considering this, the increase in adenyl cyclase activity is not very large and it is actually brought under control very rapidly. The level of cyclic AMP begins to decrease rapidly. Some of it is no doubt becoming complexed to its receptor protein (inactive protein kinase) and some may be hydrolyzed by phosphodiesterase, if there is any present. These two enzymes could account for a good portion of the disappearing cyclic AMP, but although this is happening it is not being replaced by more cyclic AMP, even with so much isoproterenol in the tissue. The controlling effect is mainly on the level of catecholamine in the tissue, i.e. on the activator of adenyl cyclase activity, rather than on the cyclic AMP itself.

Because of this rapid induction of COMT to control activation of adenyl cyclase and formation of cyclic AMP it is even possible that any buildup of measurable cyclic AMP is not the normal situation in cells: it appears to be an artificial situation resulting from over-stimulation of β-adrenergic receptors in a gland which is normally controlled by neural reflexes

acting through the autonomic nervous system. The lungs responded to isoproterenol with a similar, but smaller, increase in cyclic AMP level (Guidotti et al.,1972). They have sympathetic innervation and to measure COMT activity would be of interest.

B. CATECHOL O-METHYL TRANSFERASE INDUCTION

The increase in COMT activity after isoproterenol occurs so rapidly that it reminds one of the classical case of induction of β-galactosidase activity (described in most biochemistry texts) which occurs within 10 min after E. coli are placed in a medium containing only lactose. According to the hypothesis of Jacob and Monod (1961), all inducible enzymes are specified by genes. The genes specifying the synthesis of induced enzymes may be repressed or induced, as a group, because their synthesis is coded by a set of consecutive genes in DNA, called an operon, which can be repressed or derepressed together. The substrate may act as a derepressor and within 2 min the synthesis of new messenger RNA begins. However, this type of induction has been proven to occur only in procaryotic cells; enzyme induction and repression in vertebrates occurs for the most part, only in liver. So how does the greatly increased activity of salivary gland COMT come about?

According to the textbooks, there are believed to be only three mechanisms by which hormones can act in their target tissues: (i) by the stimulation of synthesis of particular RNA; (ii) by stimulating the adenyl cyclase system and increasing

the concentration of cyclic AMP, and (iii) by alteration of transport rates through cell membranes. Do any of these mechanisms apply to the dramatic increase in COMT activity observed so soon after isoproterenol injection?

Certain hormones of vertebrates have been shown to function by increasing the transcription rate and thus the amount of messenger RNA specifying the synthesis of an enzyme. For example, the adrenal corticosteroids cause a large increase in tryptophan pyrrolase activity in liver, which can be inhibited by actinomycin D. This type of enzyme induction (adaptation to a new metabolic situation, such as starvation) usually occurs only over a 24 hour period; thus, the increase in COMT activity is too rapid for de novo synthesis of messenger RNA to occur at the transcriptional level. Synthesis of new enzyme protein at the translational level (on pre-existing messenger RNA) is also a possibility and should be investigated further, although, at the dose of puromycin used herein, no effect on COMT levels was observed, so translational-level control is not indicated.

Let us consider the next mechanism, the formation of cyclic AMP. If an increase in cyclic AMP is causing an increase in COMT activity then the COMT molecule may have a modulatory subunit (similar to that of protein kinase) or even a modulatory site on the enzyme which induces the proper conformation of the enzyme protein to allow O-methylation of catecholamines to proceed. This type of activation would be a type of negative

feedback,(a mechanism ubiquitous in biological processes),in the sense that cyclic AMP by activating COMT would prevent adenyl cyclase activation and hence its own formation. Possibly COMT is activated by cyclic AMP in the same way as protein kinase, by dissociation of an active catalytic subunit.

Although hormonal action is primarily through the mechanisms listed above, this does not preclude secondary effects occurring, such as modulation of the enzyme activity by the product, 3-methoxyisoproterenol, or even by the substrate, isoproterenol. It is difficult to say what, exactly, is the mechanism of induction without further testing, using the possible activators mentioned or higher doses of protein synthesis inhibitors.

C. PROTEIN KINASE

Within 1 hour after isoproterenol injection, significant increases in protein kinase activity of the total homogenate, soluble fraction and nuclear fraction are observed in parotid gland (Table XI). There is evidence that increases occur in the other particulate fractions between 2 and 6 hours after isoproterenol also (Figure 19).The increase in protein kinase activity of the nuclear fraction correlates well with an increase in the acidic (nonhistone chromatin) proteins observed by Stein and Baserga (1970) and will be considered as a possible control mechanism. Ishida and Ahmed (1973) observed an increase in phosphoproteins of submandibular nuclei to occur between 2 and 24 hours after a very large dose of isoproterenol. The increase noted here

reaches a maximum at 1 hour and then decreases, but only temporarily: a second increase begins between 2 and 6 hours which may be similar to that observed by Ishida and Ahmed (1973).

PART III: POSSIBLE CONTROL MECHANISMS

A. CONTROL OF CYCLIC AMP FORMATION BY COMT

This topic has already been discussed at length in Part II. As noted, the decrease in cyclic AMP level in parotid gland is inversely proportional to the increase in COMT activity which is observed following an i.p. injection of isoproterenol.

In a gland which is controlled by neural reflexes from the autonomic nervous system, it makes very good sense to find a catecholamine deactivator, an enzyme which would normally control untimely, wasteful release of stored α-amylase and other salivary enzymes at the wrong time. If α-amylase were excreted every time a rat glimpsed one of its predators (increasing its adrenaline blood level for "flight or fight") then the parotid gland might be totally depleted of α-amylase by the time it found some carbohydrate-containing food. Thus, control of circulating catecholamines by COMT may be a conservation measure.

A control mechanism, such as this, may not be limited to salivary glands. It may be a general mechanism present in many tissues which contain sympathetic nerve endings and β-adrenergic receptors, but which are not target organs for circulating catecholamines, and there are many such tissues. This would provide,

therefore, the coarse control over the prevention of inadvertent production of cyclic AMP caused by changing levels of circulating adrenaline and noradrenaline. These tissues would therefore require only small amounts of phosphodiesterase for fine control over excess cyclic AMP under normal conditions. In this way, excretion in salivary glands can be controlled entirely by neural reflexes normally and when that once-in-a-lifetime dose of isoproterenol is administered, the glands are quite capable of controlling it, as well, although it takes a few minutes longer because of the unphysiological amount of circulating catecholamine.

B. CONTROL OF SALIVARY GLAND FUNCTIONS BY PROTEIN KINASE

1. Possible role of nuclear phosphoproteins

The immediate increase in phosphorylation of nuclear proteins was unexpected. Could it be that nuclear histones are being phosphorylated and performing a regulatory action on the cell genome, such as modifying the DNA-histone interactions and thus causing derepression of template activity? This was suggested to be their function by Stevely and Stocken (1966). Other workers have shown that actinomycin D during the first few hours after isoproterenol prevents the synthesis of DNA which occurs after 20 hours in salivary glands (Sasaki et al, 1969; Ekfors & Barka, 1971). Early effects of actinomycin D on protein kinase should be tested. Histones have also been reported to cause a 5- to 10-fold

increase in the rate of phosphorylation of nuclear (nonhistone acidic) phosphoprotein without being phosphorylated themselves (Kaplowitz et al, 1971). These phosphorylated acidic proteins have been shown to bind to DNA and cause enhancement of RNA synthesis (Teng et al, 1971) possibly by displacing histone from the DNA template during the process of gene activation (Klein-smith et al, 1966).

The synthesis of these acidic nuclear proteins was shown to increase (by incorporation of ^{3}H-leucine) within 10 min after isoproterenol in mouse salivary glands (Stein & Baserga, 1970). This increase in acidic nuclear proteins was not inhibited by actinomycin D, so they suggested that the synthesis of acidic nuclear proteins must be controlled at the translational level. The increased protein kinase activity in the nuclear fraction, found in the present studies, was also shown to be inhibited by puromycin at 2 hours (Figure 19). It would be quite a coincidence if the proteins being phosphorylated were not the acidic nuclear proteins. Of course, this implies an increase in the amount of substrate, as well as an increase in active protein kinase; maybe both occur. A great deal of work remains to be done on immediate effects of isoproterenol on nuclear proteins in salivary glands. Ishida and Ahmed (1973) report a decrease in submandibular nuclear phosphoproteins at 2 hours and an increase between 2 and 24 hours relating to DNA synthesis. The immediate increase, observed herein, may be related to formation

of new RNA templates for the synthesis of proteins involved in hypertrophy and hyperplasia.

2. Possible control of α-amylase synthesis

Resynthesis of α-amylase was reported to be in progress within 1 hour after adrenaline stimulation of rat parotid glands and to continue for several hours (Grand & Gross, 1969). They suggested that it is controlled at the translational level because they found α-amylase being synthesized in the absence of RNA synthesis (Grand & Gross, 1970). The pattern of protein kinase activity in the soluble fraction and the effects of protein synthesis inhibitors in the present studies are the same as those found for α-amylase. Protein kinase of the soluble fraction has been found to phosphorylate 40S and 60S ribosomal subunits in liver (Eil & Wool, 1971). Thus, a relationship between protein phosphorylation and α-amylase synthesis may occur at some step of the amino acid incorporation into α-amylase. On the other hand, Gaunce (1971) could find no immediate precursor of α-amylase to be activated by phosphorylation with protein kinase.

3. Possible role in the secretory process

The crude nuclear fraction in which increased protein kinase activity was observed (Table XI) contained fragments of cell

membranes, and possibly membranes of broken zymogen granules, as well. Hokin and Sherwin (1957) reported that adrenaline can elicit an increase in the rate of ^{32}P-incorporation into phospholipids, as well as cause protein secretion. They observed increased turnover of phospholipids during induction of secretion in rabbit salivary glands. Myoinositol-2-^{3}H was recovered in zymogen granules of pancreas stimulated to secrete by pilocarpine. Hokin suggested a model (1968) in which phosphatidyl inositol links together lipoprotein subunits in the membrane surrounding secretion granules. A cyclic AMP-stimulated protein kinase may thus take part in phosphorylation of membrane phospholipids. The rapid turnover in ^{32}P may result from the action of kinase and phosphatase which have been shown to exist in dynamic equilibrium by Weller and Rodnight (1971).

CONCLUSIONS AND SUMMARY

The catecholamine, isoproterenol, induces changes in all of the three enzymes studied, catechol O-methyl transferase, adenyl cyclase and protein kinase, as well as an amazing decrease in the protein content of the parotid gland.

1. Protein

It is concluded that because of the drop of over 50% in the protein content of parotid gland (and smaller but definite changes in the submandibular gland), any studies in rodent salivary glands which report increases in the specific activity (activity per mg protein) between 10 min and 12 hours after isoproterenol treatment, should be viewed with suspicion.

2. Adenyl cyclase

Isoproterenol is shown to produce increased activity of adenyl cyclase and an increase in cyclic AMP levels in parotid gland. Because the salivary glands are known to be controlled by neural reflexes, only noradrenaline, released from the sympathetic nerve endings, may be acting on β-receptors *in vivo* to produce cyclic AMP (as well as its action on α-adrenergic receptors). As soon as cyclic AMP is formed it may combine with its receptor protein, the inactive protein kinase. No circulating catecholamines may have the opportunity to stimulate β-adrenergic receptors because they are inactivated by the catechol O-methyl transferase which is rapidly induced in their presence.

The adenyl cyclase activity of parotid gland can be stimulated by EGTA which chelates the endogenous Ca^{2+}. This finding may be significant because Ca^{2+} is essential for the excretion of α-amylase and is present in higher concentration in salivary glands than in other soft tissues.

3. Catechol O-methyl transferase

COMT activity is very high in rat salivary glands and is located in both the particulate and soluble fractions. An increase is noted within 10 min after isoproterenol injection. The activity triples by the end of 1 hour and then decreases again. This increase is inversely proportional to the decrease in cyclic AMP level which was noted after 10 min. Thus, an induction in COMT activity may control the amount of circulating catecholamine which activates adenyl cyclase and hence COMT may control formation of cyclic AMP. It is suggested that this may be a general control mechanism operating in all tissues with β-adrenergic receptors, which are not target organs for circulating catecholamines, in order to prevent cyclic AMP formation by catecholamines, other than the neurotransmitter, noradrenaline in tissues with sympathetic innervation.

4. Protein kinase

a) The protein kinase of salivary glands, like that of many other tissues, consists of a regulatory and a catalytic subunit. Activation occurs by binding of cyclic AMP to the inactive complex, causing dissociation of the active catalytic subunit.

b) The protein kinase of salivary glands is distributed between the soluble and particulate fractions. The particulate fractions are demonstrated to contain latent activity which can be released by the detergent Triton X-100.

c) Isoproterenol acts through the adenyl cyclase system to increase protein kinase activity in rat parotid gland. Conditions employed were designed to inhibit phosphatase activity. In vivo the end result may be a decrease in phosphorylated proteins if the phosphatase activity is higher than the kinase activity. The increase in protein kinase activity may thus indicate only an increased rate of ^{32}P turnover.

d) The increase in phosphorylated proteins after isoproterenol treatment may be the result of increased synthesis of a substrate of protein kinase or an increased amount of the regulatory subunit. The increase in phosphorylated protein in the nuclear fraction occurs at the same time as the increase in synthesis of acidic nuclear proteins reported by Stein and Baserga (1970). The action of the protein synthesis inhibitor, puromycin, is the same in both instances.

e) One of the natural substrates of protein kinase in salivary glands has an isoelectric point which is very similar to that of histone.

f) Isoproterenol-induced changes in protein kinase activity can be related chronologically with events in the soluble

fraction related to the synthesis of α-amylase. The effects of puromycin on the two processes are similar.

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ABSTRACT

STUDIES RELATING THE ACTION OF ISOPROTERENOL
AND THE ADENYL CYCLASE SYSTEM IN RAT SALIVARY GLANDS

The adenyl cyclase system was studied in rat salivary glands because they are excellent models in which to study the effects of catecholamines on β-adrenergic receptors. Isoproterenol was used in these studies because it is the prototype of compounds which act mainly at β-adrenergic receptors.

These investigations were initiated in an attempt to establish possible control mechanisms over the formation of cyclic AMP and its role in the sequence of biochemical events leading to known responses. Although in vitro measurement of cyclic AMP formation was difficult because the enzyme is membrane-bound and destroyed by homogenization, the tissue level following in vivo injection of isoproterenol was measured and found to increase immediately and to decline rapidly within a few minutes. Two enzymes thought to be involved in this effect were thoroughly studied. Catechol O-methyl transferase (COMT) and protein kinase were both found to have high endogenous activity in parotid and submandibular glands. The subcellular distribution was determined for each enzyme and both were found to be present in the particulate fractions as well as the soluble fractions.

Following an injection of isoproterenol, the activity of COMT was found to increase significantly within 30 min in both glands. Because its activity varied inversely with the level of cyclic AMP

in the tissue after 10 min, a control mechanism over the formation of cyclic AMP in glands which are not target organs for circulating catecholamines is suggested.

In order to assess whether protein kinase is involved in reactions coupling the second messenger, cyclic AMP, with various effector systems in the cell, it was necessary to purify the enzyme partially, and to determine its characteristics and mechanism of activation. In addition, attempts were made to identify its natural substrate.

Effects of the protein synthesis inhibitors, actinomycin D and puromycin, were studied. Actinomycin D had no inhibitory effect on either enzyme but puromycin appeared to inhibit protein kinase activity, or possibly one of its substrates, at certain intervals after isoproterenol injection, in some of the subcellular fractions.

Taking precautions to avoid the action of phosphatases, it could be shown that an injection of isoproterenol caused an increase in the endogenous protein kinase activity in some subcellular fractions of parotid gland. The increase in phosphorylation of proteins could be related chronologically with functions known to occur in the main organelles of the fractions. Therefore, it is suggested that phosphorylation of another enzyme (or protein) may play a role in at least one of the sequence of steps between catecholamine binding at the β-adrenergic receptor and the varied responses of the parotid gland.