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CYCLIC AMP AND LABILE HYPERTENSION

A Micheline

IMPLICATION OF CYCLIC AMP IN THE PHYSIOPATHOLOGY OF LABILE HIGH BLOOD PRESSURE.

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LIST OF ABREVIATIONS

ACTH	:	adrenocroticotrophic hormone
ADH	:	antidiuretic hormone
ADP	:	adenosine diphosphate
5'-AMP	:	5'-adenosine monophosphate
ATP	:	adenosine triphosphate
CL_{95}	:	confidence limit for 95% of significant level
CPM	:	counts per minute
Cyclic AMP	:	3', 5'-adenosine monophosphate
db-cyclic AMP	:	dibutyryl derivative of 3', 5'-adenosine monophosphate
DEAE	:	diethylaminoethyl cellulose
df	:	degrees of freedom
DOCA	:	deoxycorticosterone
DPM	:	disintegrations per minute
EDTA	:	disodium ethylenediaminetetraacetate
F	:	value of the analysis of variance or covariance
MBP	:	mean blood pressure
MS	:	mean square
NADPH	:	dihydronicotinamide adenine dinucleotide phosphate
NS	:	non significant at the level of 95%
p	:	probability
PG-	:	prostaglandin
S^2	:	variance
SE	:	standard error of the mean
t	:	value of Student test
T	:	value of Wilcoxon test

Motto: *Since cyclic AMP is involved in many important regulatory events but may not be essential for life (at least not in the sense that such factors as the calcium ion and ATP are essential), it seems likely that we will see a very large number of defects in the cyclic AMP system in man.*
(Earl W. Sutherland, 1970) (259).

I - INTRODUCTION

The etiology and the physiopathological mechanism of essential hypertension is, in 1972, still unknown. Many groups of workers, particularly that of Genest, Küchel, Nowaczynski and Boucher, have contributed to a better insight of humoral disturbances in this entity by their studies on renin-angiotensin-aldosterone system, mineralocorticoids, progesterone, catecholamines, and other parameters.

The secretion and/or action of many of these blood pressure regulating substances was shown to be mediated through the system of adenyl cyclase-cyclic AMP. This mediatory mechanism is a part of more general informational activity of cyclic AMP. The rapidly growing area of "second messenger system" was recently reviewed by leading workers, Robison, Butcher and Sutherland, in their monography *Cyclic AMP* (221).

The work presented in this thesis was undertaken as a first approach in search for disturbances in the transmission of hormonal information in essential hypertension. A special attention was given to patients with labile hyperkinetic hypertension, since it is believed that they are precursors of stable essential hypertension. In this study, we were able to distinguish control subjects from patients with labile hyperkinetic hypertension because the excre-

tion of cyclic AMP in response to the stimulation by upright posture or isoproterenol was different for the two groups. The propranolol treatment tended to abolish the differences in certain patients.

Thus, the present study is concerned with two vast areas, that of hypertension and that of cyclic AMP. Only the investigations with a potentially interrelated significance are therefore discussed in the review of literature.

II - REVIEW OF LITERATURE

A - ESSENTIAL HYPERTENSION

1 - DEFINITION

Essential hypertension is a "disease" representing the consequence of raised blood pressure without any evident cause (206). Two major views are discussed in the literature: the first one, represented by Pickering, suggests that high blood pressure is a disease of degree and not a disease of kind, or quantitative not qualitative (206). This conclusion is based on the observation that, in a large population, blood pressure values are distributed continuously without any definite sign of delimitation between normal and abnormal groups (28). The second group of authors, represented by Platt, demonstrated evidence for a qualitative character of this disease. This evidence is based on the observation that the distribution of blood pressure values in siblings of severe hypertensives does not correspond to the Gaussian curve, as it should, if high blood pressure was simply a graded characteristic with a multifactorial inheritance (208).

2 - LABILE AND STABLE TYPE OF HYPERTENSION

Any division of population, normal and abnormal, on the basis of blood pressure, is of necessity highly arbitrary. More difficult and controversial is the separation of a hypertensive population into subgroups. Such separation nevertheless offers many experimental and clinical advantages. Today most workers, for practical purpose, use as a dividing line between normal and abnormal the pressure of 140/90 mmHg. It is more difficult to establish

a dividing line between labile and stable types of hypertension. Sannerstedt et al. defined as labile, or borderline, those hypertensive patients having at least one out of five auscultatory readings over 90 mmHg and at least one below 90 mmHg diastolic pressure (230). Frohlich et al. defined labile hypertensive patients as those whose arterial pressure was elevated on several occasions, with many intervening periods in which normal arterial pressure was measured (80).

Genest's group used for the selection of patients with stable and labile benign essential hypertension the following criteria: "normal" physical examination and absence of retinopathy, normal serum Na^+, K^+ and total CO_2^{--}, normal renal function as measured by serum urea and creatinine, normal phenolphthalein excretion, creatinine clearance, and rapid sequence intravenous pyelography; normal electrocardiogram and renal arteriography; absence of any overt signs of arterio-atherosclerosis of large vessels. The patients selected according to the above criteria are then divided into two subgroups: one with blood pressure readings always above 140/90 mmHg at the time of study is classified as having stable benign essential hypertension. The second group with blood pressure readings decreasing to below 140/90 mmHg during hospitalization is classified as having labile essential hypertension (84). A large portion of patients from the latter group present symptoms and signs of hyperkinetic circulation such as: palpitations, usually elevated heart rate at rest, with a constant exaggerated response to posture or cold, and often some degree of correlation between heart rate and blood pressure. These patients also showed a high incidence of symptoms of neurovegetative lability with, in

decreasing order of frequency: dermographism with or without macular erythema, acrohypothermia and hyperhidrosis, anxiety and emotional lability and dyspepsia (137). The above definitions are used throughout the work presented in this thesis (refer section of Materials).

Widimský et al. showed for the first time in 1957 that juvenile subjects with labile essential hypertension, which they considered to be the early stage of essential hypertension, frequently had high cardiac output (284). Later in 1966, Frohlich called this state of elevated cardiac output the "hyperdynamic β-adrenergic circulatory state" (79). In still another study, Sannerstedt (230) observed a high cardiac output in patients with hyperkinetic circulation as well; however, the change due to the tilt was of the same magnitude in patients as in control subjects. Küchel et al. reported an exaggerated response in plasma renin activity to upright posture in this type of patient (137).

It is debatable whether or not these patients with labile hypertension and hyperkinetic circulation present a precursor stage of a stable form of essential hypertension. Evelyn analyzed data from the Metropolitan Life Insurance Company of Canada containing results of medical examinations carried out over periods of 25 to 35 years (67). It is evident from this study of prolonged observations, that one cannot predict the issue of elevated blood pressure which may be present for a period of time in the patient's life. It is obvious that both issues, a definite hypertension or normotension, are possible. In a study of patients with labile hypertension, Eich et al. observed that about half of patients with labile hypertension were characterized by a high cardiac output and normal or low peripheral resistance (61). When the follow up in this prospective study was performed fifty months later, the workers ob-

served a diminution of cardiac output and an increase in peripheral resistance, i.e. the tendency toward the state usually observed in the stable form of essential hypertension (normal cardiac output and high peripheral resistance). Since the study was performed on an out-patient basis, they could not reevaluate the "lability" of hypertension after the follow up period. The group of Brown also suggested that labile hypertension is a preliminary phase in high blood pressure physiopathology (29). According to this group, the transition from the labile to persistent form of hypertension is characterized by a change from increased cardiac output to an increased total peripheral resistance.

Since β-adrenergic hyperactivity may be an underlying cause of hemodynamic disturbances in this type of patient , the use of β-blocking agents would be a logical approach for pharmacotherapy in these subjects. The most popular β-blocking agent, L-propranolol, has been shown to be useful in the therapy of hypertension (81). It diminished cardiac output and increased peripheral resistance. Reduction in the mean arterial pressure with this treatment appears to be directly related to the height of resting pretreatment cardiac output (80). Propranolol was proved to be useful in the treatment of anxiety amongst patients whose symptoms are due to adrenergic β-receptor stimulation (92). This beneficial effect was obtained only with the L-form of the drug (20).

3 - EFFECT OF POSTURE ON BLOOD PRESSURE REGULATION

Many hemodynamic and excretory modifications, secondary to the change of body position, were described in the past century. The first report, accredited to Piorry in 1826, describes the effect of body position on cerebral cir-

culation in human and bled animals (207). The adjustment mechanism for the "standing still" position appears to be an increase in vaso-constrictor tone in the dependent portion of the body. Thompson (266) studied in 1928 the relation of body position to blood volume. Epstein et al. showed in 1951, that the well-known antidiuretic reaction to standing does not require a contraction of the total plasma volume, and that the volume expansion by hypertonic saline was able to reverse this antidiuretic reaction (64). "Quiet standing" was also accompanied by a diminution of sodium excretion, glomerular filtration and, to some extent, changes in the excretion of potassium and urinary pH (65).

Sundin measured urinary excretion of catecholamines (258) in search of a mechanism of hemodynamic adaptation during upright posture. The excretion of norepinephrine increased substantially in the 75^0 tilted position, whereas that of epinephrine increased only moderately or remained unchanged. This reaction to posture was largely abolished by a ganglion-blocking agent.

Plasma renin activity increased significantly during adaptation to the upright position (45). This is not surprising if we consider that the regulation of renin is related morphologically and functionnally to the activity of sympathetic nervous system (225) (91).

Horký et al. studied the sympatho-reno-adrenal system in response to postural adaptation (109). They observed during upright posture an increase of the leg volume, a drop of systolic and a rise of diastolic blood pressure, accompanied by an increase in pulse rate, norepinephrine excretion, plasma renin activity, aldosterone excretion and a drop in excretion of water and electrolytes. These changes could be prevented by bandaging of lower extremities and

so preventing the shift of body fluids. Küchel et al. performed a qualitative analysis of urinary catecholamines during postural adaptation and studied their relation to the renin-angiotensin system in normotensive and hypertensive subjects (135). (The results of this study are considered in the discussion part of this thesis).

B - CYCLIC AMP INVOLVEMENT IN THE SECRETION OR ACTION OF SUBSTANCES REGULATING BLOOD PRESSURE

1 - GENERAL DEFINITIONS

The discovery of *cyclic AMP* in 1956-57 was the consequence of the biochemical work of Sutherland and Rall and a chemical work of Cook, Lipkin and Markham (260, 48). The former group identified this nucleotide as a heat-stable factor (formed by particulate fractions of liver homogenates in presence of ATP, Mg^{++}, and epinephrine or glucagon) stimulating the formation of phosphorylase in supernatant fractions of homogenates. The latter group isolated independently this particular nucleotide from a barium hydroxide digest of ATP and identified the chemical structure as being the mononucleotide, adenosine-3', 5'-phosphoric acid.

The formation and metabolism of cyclic AMP is mainly regulated by two enzymes. The first one is a membrane bound *adenyl cyclase* (or, as recently called adenylate cyclase) (220). This enzyme catalyzes the conversion of ATP to cyclic AMP in the presence of magnesium ions. The second is a *phosphodiesterase* which catalyzes the breakdown of cyclic AMP to 5'-AMP (33). From the work of Sutherland, Rall, Butcher, Robison and many others, it became evident that many hormones may be regarded as a first messenger which carries

the information to adenyl cyclase. The so-activated enzyme accelerates the catalysis of ATP to cyclic AMP. In turn, cyclic AMP serves as the *second messenger* transporting the original hormonal information into the cell machinery.

It is now quite generally accepted that cyclic AMP itself acts by interaction with another enzyme, the *protein kinase* (85). The receptor subunit of this enzyme dissociates after the binding of the nucleotide and thus the catalityc subunit may realize its function. This function is the phosphorylation of various substrates such as histones, ribosomes or caseine. It is to be noted that cyclic AMP bound to the protein kinase seems to be protected from the hydrolysis by the phosphodiesterase (192). There is more and more evidence that the regulation of the intra-cellular level of cyclic AMP is a complex framework of different mechanisms. We may mention as an example the regulatory influence of another natural cyclic nucleotide, cyclic GMP, which is able to influence the activity of the phosphodiesterase and so affect the catabolism of cyclic AMP (98). Another pertinent example comes from the work of Ho et al. and Manganiello et al. (107, 170) who presented the evidence that a quick increase of cyclic AMP in cells, resulting from hormonal stimulation, is rapidly followed by the production of an antagonist of cyclic AMP. A possibility of a negative feedback loop is so evcked.

A frequently asked question by general medical public is the so-called "unspecificity" of the ubiquitous cyclic AMP, contrasting with hormonal "specificity". The problem is without foundation since adenyl cyclase, the cyclic AMP generating enzyme, is in itself a guaranty of specificity. The enzyme of

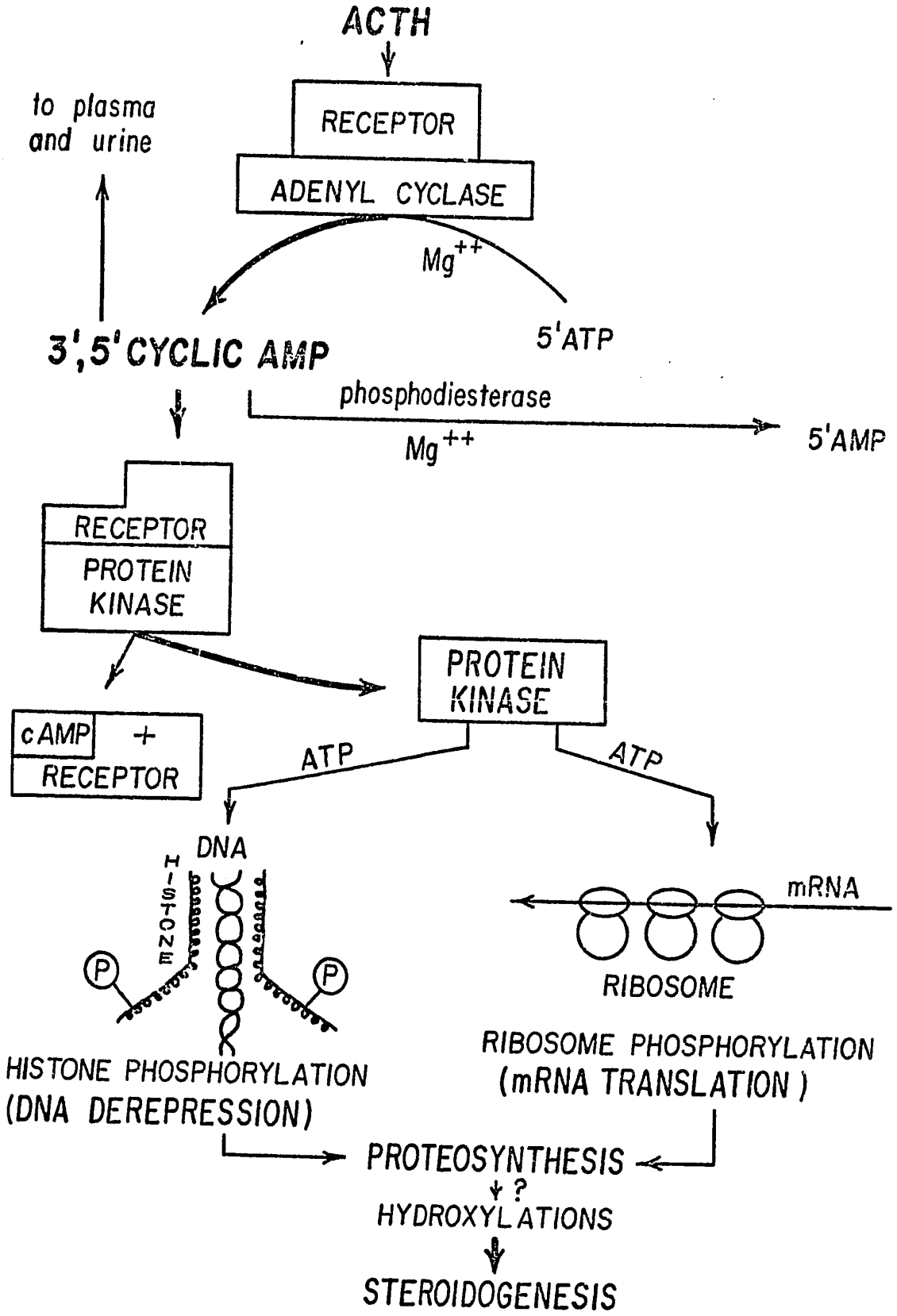


Fig. 1 Schema demonstrating the transmission of ACTH information to the adrenal cortex cell.

each cell acts with different hormones in a specific manner, as shown for example by the interaction with hormones and their antagonists (e.g. β-blocking agents) (221). However, as will be discussed later in this chapter, a loss of this specificity was observed in endocrine neoplasic tissue (190). On the basis of the results of many workers, a tentative schema of the action of cyclic AMP is presented in an example chosen from the transmission of the information induced by ACTH (fig. 1).

2 - CATECHOLAMINES

a - *Adrenergic receptors*

The concept of adrenergic receptors is mainly based on the dual receptor theory of Ahlquist (3). This concept replaced the unsatisfactory terminology of "excitatory" and "inhibitory" of Cannon (34) with the introduction of terms alpha and beta for distinction of receptors. Ahlquist's theory received many criticisms, but its practical use is widespread (8). The practical usefulness of separation of receptors into α- and β-categories is concerned with pharmacological application of the effects of α- and β-blockers.

During the past decade, many workers have accumulated evidence of the involvement of cyclic AMP system in the "field" of adrenergic receptors. A correlation between β-activation and the rise of cyclic AMP in cells of cardiac muscle (187), smooth muscle (270), kidney (181) and other tissues was shown in many studies. The β-activity and rise in the level of cyclic AMP may be inhibited by so-called β-blocking agents (187, 226). On the basis of these findings, Robison suggested that β-receptors and adenyl cyclase are integral components of the same system (218, 221).

The situation is far more complicated for the α-receptor. It was originally suggested that the α-activity is related with the fall of cyclic AMP level as a consequence of inhibition of adenyl cyclase activity by α-agonists. This is true in some tissues such as platelets (218, 229), but other studies bring up conflicting results. Alpha-activation may interfere with both formation and action of cyclic AMP (71); in brain tissue, however, cyclic AMP may be increased by both α- and β-stimulation (40). It must also be pointed out that catecholamines may act elsewhere than on adenyl cyclase. Sheppard and Wiggan demonstrated recently that 3,4-dihydroxyphenylacetic acid and apomorphine are inhibitors of phosphodiesterase and that therefore, this cyclic AMP catabolizing enzyme may be an important component of a "dopamine receptor" (243).

b - *Effect on heart muscle*

The action of catecholamines on heart muscle is mainly characterized by their positive chronotropic and inotropic effects; the latter was related to phosphorylase activation in 1959 by Kukovetz (142). The same author in 1968 (141), during the perfusion of dibutyryl derivative of cyclic AMP in a guinea pig heart, observed the inotropic and chronotropic responses as well as phosphorylase activation. Many other studies led to the conviction that at least the β-component of inotropic response is mediated through cyclic AMP (157, 54, 219). Studies on heart muscle metabolism also suggest the regulatory role of cyclic AMP (27). Another study on the influence of phosphodiesterase blocking agents does not support the concept that cyclic AMP is a common mediator of mechanical and metabolic functions in the heart (126).

Calcium accumulation by sarcoplasmic reticulum, related with contractile activity in the heart, is increased by epinephrine, glucagon and cyclic AMP (66, 245). The work of Epstein's group is of particular interest: in their experience, glucagon and epinephrine required a longer incubation time before the calcium accumulation was observed than was required for cyclic AMP itself, which was shown to have an immediate effect. This finding, concluded Epstein, might be related to the mechanism of action of epinephrine and glucagon. The proposed mechanism would be that an initial increase in concentration of cyclic AMP must first be produced through adenyl cyclase stimulation before the effect of epinephrine and glucagon on calcium accumulation appears. Further observations on effect of glucagon and calcium are discussed in sections II-B-5 and II-B-7-a.

Adenyl cyclase activity has also been measured in acute hemodynamic overload in perfused guinea pig heart. The total adenyl cyclase activity was significantly increased in the particulate fraction obtained from the overloaded left ventricles (237). This finding is of particular interest especially when correlated with a diminution of activity in some specialized receptor areas of adenyl cyclase in heart insufficiency (see glucagon, part II-B-5-a).

The mediatory role of cyclic AMP has been also discussed in relation to isoprenaline-induced cardiac necrosis in the rat (172). The dibutyryl derivative of cyclic AMP induced myocardial lesions of the same type as the β-activator. Moreover, the tension activity induced by isoprenaline was highly increased in vivo by pretreatment with theophylline which was shown to inhibit the degradation of cyclic AMP.

c - *Effect on vascular smooth muscle*

Somlyo and Somlyo on the basis of results from many laboratories concluded that, with a few exceptions, a rise in intracellular cyclic AMP was generally associated with smooth muscle relaxation (251). Some workers, however, were unable to influence the aortic adenyl cyclase activity with isoprenaline or norepinephrine. It is possible that this failure may be a result of technical difficulties (235). Volicer and Hynie, in a more fruitful study, evaluated the activity of aortic adenyl cyclase by measuring the enzymatic activity and incorporation of labelled cyclic AMP using adenine marker as a precursor (272). Their results are consistent with the hypothesis that vasodilatation (through β-activation and/or α-blockage) is associated with an increase and vasoconstriction(through α-activation and/or β-blockage) with a decrease in the cyclic AMP level in vascular smooth muscle. The β-adrenergic hyperpolarization of the main pulmonary artery, mediated through cyclic AMP, was shown to be dependent upon a low concentration of potassium (249), (see also part II-B-7-a).

Papaverine was demonstrated to be a very potent inhibitor of the activity of phosphodiesterase (143). Stoclet et al. studied the inhibitory effect of papaverine on isotonic contraction of aortic strips induced by barium (253). They showed that aortic strips were more sensitive to papaverine in rats with DOCA induced hypertension than strips from untreated animals. Strips from animals treated with DOCA which did not develop hypertension, presented a papaverine sensitivity situated somewhere between that of normal and hypertensive animals. Berti et al. (14), in a study of a direct effect of cyclic AMP and db-cyclic AMP on vascular reactivity, demonstrated that db-cyclic AMP induced

relaxation during *in vitro* perfusion of rat caudal artery. Theophylline duplicated the effect of db-cyclic AMP, and imidazole had an opposite effect while infusion of cyclic AMP itself induced an increase in the tonus of the smooth muscle. This adenosine like effect may be due to the rapid destruction of cyclic AMP by phosphodiesterase during the perfusion procedure.

d - *Effect in nervous tissue*

Blood pressure is, at least in part, controlled by the central nervous system (131, 52). It is important therefore to note that many blood pressure regulating substances have been shown to modify the activity of adenyl cyclase in the central nervous system (93). Adenyl cyclase activity and the level of cyclic AMP increase markedly after birth in rat brain (233). Adenyl cyclase in cerebral cortex is sensitive to norepinephrine and dopamine (177). Histamine and serotonin were reported having activity which could be considered "more than additive" to that of catecholamines (110). Schmidt, studying the specificity of brain adenyl cyclase, observed the typical β-adrenergic receptors in cerebellum, while the cerebrum contained the α-receptors (234). As previously mentioned (refer chapter II-B-2-a), both α- and β-adrenergic stimulation can increase the level of cyclic AMP. Furthermore, the activation of cerebellar adenyl cyclase mediated by norepinephrine seems to be also modulated by endogenous prostaglandins (247).

McAffee showed in superior cervical sympathetic ganglia from rabbits the ability of a brief supramaximum stimulation to increase cyclic AMP concomitantly with the synaptic transmission (176). Siggins reported that norepinephrine and cyclic AMP are both able to hyperpolarize cerebellar Purkinje cells and

elevate transmembrane resistance (248). A suggested conclusion that may be drawn from the aforementioned and other observations is that cyclic AMP provides a molecular basis for integrative actions within the nervous system (93).

Krishna measured adenyl cyclase and phosphodiesterase activities concomitantly in many areas of cat brain in order to evaluate the dynamics of neurohormonal control of cyclic AMP synthesis (133). Apparently, in those areas of the brain which contain the highest concentration of catecholamines, the ratio of adenyl cyclase to phosphodiesterase is lower than in areas which have a low concentration of catecholamines such as the cortex, cerebellum and inferior colliculus. The importance of his results remains to be established.

Circulatory changes induced by various stress stimuli were observed in laboratory animals as well as in humans (75, 25). Paul reported recently a significant increase of cyclic AMP in adrenals of rats submitted to immobilization stress (202) and Therriault et al., the stimulatory effect of the cold on the adenyl cyclase reactivity to norepinephrine (265). In contrast, hemorrhagic shock decreased cyclic AMP in liver (228).

3 - RENIN-ANGIOTENSIN-ALDOSTERONE

a - *Renin release from kidney*

The regulation of renin release is, at least in part, controlled by the sympathetic nervous system. Direct sympathetic innervation was shown by the electron microscopic studies of Barajas in monkey and of Ortega et al. in rat and human juxtaglomerular apparatus (9, 224, 225). Gordon et al. observed an increase of plasma renin activity in response to either the infusion of catecholamines or the stimulation of the sympathetic nervous system by cold (91). A rise in plasma renin activity is known to be induced by upright posture (45),

a response believed to be mediated by the sympathetic nervous system (258). Michelakis et al. studied the effect of catecholamines *in vitro*, using a system of kidney cell suspension to eliminate any hemodynamic influence, and showed that the addition of cyclic AMP had a similar stimulating effect as catecholamines on the release of renin (185).

There are three studies concerned with the effect of cyclic AMP on renin secretion *in vivo*. The first from Winer et al. demonstrates that cyclic AMP is a potent stimulant of renin secretion (287). Disturbing is however, that both α- and β-blocking agents were able to abolish the stimulation of renin secretion induced by the infusion of cyclic AMP. A tentative conclusion would be that blocking agents can influence hormonal activity after formation of cyclic AMP as well, i.e. by influencing its metabolism and/or act at its site of action (protein kinase receptor site ?). The non specific effect of α- and β-adrenergic blocking agents on the action of cyclic AMP was also noted by Schreibman et al. in adipose tissue lipolysis (238). Tagawa and Vander in a study contradictory to that one of Winer (264) reported that they were unable to demonstrate the stimulation of renin secretion by cyclic AMP and even observed a diminution of renin with very high doses of cyclic AMP. This discrepancy may be due, as these workers suggest, to the fact that their study was conducted in salt-depleted dogs where the renin was already maximally stimulated and therefore a relatively small increase may not have been evident. In a third study from Hauger-Klevene (99), it was demonstrated that the stimulation of renin in rats was achieved by administration of ACTH. Cyclic AMP induced a similar rise in plasma renin level and in addition theophylline potentiated the effect of ACTH on this release. Dexamethasone administration

abolished the stimulating effect of both ACTH and cyclic AMP on renin release. This inhibitory effect was abolished by pretreatment with actinomycin D and therefore presumably involved RNA synthesis (DNA dependent).

b - *Action of angiotensin*

Angiotensin II has three major effects. The first one is its effect on the contractibility of the smooth muscle (30, 56). The second one, described for the first time by Genest et al. (82) and shortly afterward by Laragh et al. (152), was the stimulation by angiotensin of aldosterone excretion and secretion rate. The third effect involves the autonomic nervous system and it includes the parasympathomimetic effect, the activation of sympathetic ganglia, the release of catecholamines by adrenal medulla, and the sympathomimetic effect due to its action on the brain (167). Only the first two of the above effects of angiotensin have been studied in relation to cyclic AMP until now.

aa- <u>action on the vascular smooth muscle</u>

Volicer and Hynie in 1971 reported for the first time the influence of angiotensin on the accumulation of labelled cyclic AMP in the arterial wall of rats (272). Angiotensin in presence of theophylline produced a significant decrease of cyclic AMP level in the tail artery and aorta. The activity of aortic adenyl cyclase was significantly decreased by angiotensin in opposite to the rise induced by isoproterenol.

bb- <u>action on steroidogenesis</u>

In the study of Kaplan, cyclic AMP added to the incubation media produced a rise of aldosterone biosynthesis in the outer cortex of beef adrenal tissue to the same degree as angiotensin (119). The author concluded however that

angiotensin does not act via cyclic AMP, since it does not increase adrenal phosphorylase activity. This objection may be irrelevant since other explanations for cyclic AMP action are presently available (221) (see also II-B-4-b-dd). Aldosterone production was also stimulated *in vitro* by cyclic AMP in an incubation of rat adrenal tissue as reported by Shima et al.; however, the relation to angiotensin action was not made (244).

c - *Does the aldosterone act via cyclic AMP ?*

The effect of aldosterone on sodium transport was widely studied in *in vivo* and *in vitro* experiments in kidney and in toad bladder. There is a good deal of evidence that aldosterone induces DNA-dependent RNA synthesis. It is assumed that the new RNA serves as messenger for de novo synthesis of the so-called aldosterone-induced protein (59, 70). Edelman and co-workers suggested that this protein is an enzyme which enhances the utilization of a substrate to provide the energy needed for the active transport of sodium (60). Sharp and Leaf suggested in 1966 that this enzyme may work as a permease facilitating the entry of sodium into the cell (242). In an effort to specifically localize the effect of aldosterone on RNA synthesis, Trachewsky studied the chromatin activity in kidney of aldosterone treated rats (269) and found that the chromatin template activity did not change following aldosterone treatement. However, differences in binding of actinomycin D suggested that the physical or chemical state of chromatin was changed by aldosterone treatment.

Kirchberger et al. reported recently that the stimulation of sodium transport is accompanied by an inhibition of the hexose monophosphate shunt pathway (124). The same group in a parallel study found similarities between the ef-

fects of aldosterone and cyclic AMP on sodium transport as well as oxygen and glucose utilization (125). Both the dibutyryl derivative of cyclic AMP and cyclic AMP plus theophylline have an effect similar to that of aldosterone. The authors were unable to demonstrate a rise of cyclic AMP concentration in toad bladder tissue after prolonged incubation with aldosterone. This fact precluded the authors from infering that aldosterone actually acts via cyclic AMP. Their conclusion may be incorrect however for at least two reasons. First, the authors do not mention any precaution taken to prevent a possible destruction of increased level of cyclic AMP during sampling. This is usually achieved by ultrarapid freezing in liquid nitrogen and was shown to be essential (188). Secondly, the reliability of their cyclic AMP determination was based upon the fact that vasopressin could stimulate nucleotide concentration in their preparation, and that aldosterone did not. However, the time of incubation with vasopressin was six minutes while it was six hours with aldosterone. Cyclic AMP may have been already destroyed by phosphodiesterase after such a lengthy incubation. All this does not necessarily mean that the observed physiological effect was not induced by the initial rise of cyclic AMP.

4 - REGULATION OF ADRENAL STEROIDOGENESIS

a - *Release of ACTH*

Pituitary tissue was shown to contain adenyl cyclase activity. Both theophylline and dibutyryl cyclic AMP stimulated *in vitro* the release of ACTH in the study of Fleischer et al. (74). Dexamethasone inhibited the release of ACTH at a site distal to the action of cyclic AMP. In another study, Zor et al. demonstrated an increased formation of cyclic AMP *in vitro* by crude ovine

hypothalamic extract in the anterior pituitary gland of rat, but not in the posterior pituitary, pineal, adrenal or the liver (289). An *in vivo* study of Hedge, with stereotactic microinjection of db-cyclic AMP or of cyclic AMP with theophylline into the hypothalamus and/or the anterior pituitary of rats induced an increase of ACTH secretion (103). These findings suggest that cyclic AMP tissue level is influenced by a corticotropin releasing factor and that the nucleotide is a mediator of ACTH secretion.

b - *ACTH effect on adrenal cortex*

The fact that particulate fractions of adrenal cortex contain adenyl cyclase activity and that ACTH is able to enhance this activity, with a subsequent increase of cyclic AMP level, has been known from the start of the "cyclic AMP era". Between 1958 and 1960, Haynes and his collaborators proposed the hypothesis implicating cyclic AMP in ACTH dependent steroidogenesis (101), which was based on the following observations: 1) the rate limiting step in the steroidogenesis is the phosphorylation, permitting the breakdown of glycogen to glucose-1-phosphate; 2) phosphorylation is required for further formation of NADPH which in turn is essential for the hydroxylation of steroids; 3) the phosphorylase activity in the adrenal is dependent on cyclic AMP level. This hypothesis, although challenged today, instigated numerous studies implicating cyclic AMP in the regulation of adrenal steroidogenesis.

aa- adrenal adenyl cyclase and phosphodiesterase

Recently the adenyl cyclase activity in adrenal cortex was extensively investigated. Kelly and Koritz studying the activity of adenyl cyclase in particulate fractions of homogenate of adrenal cortex (122) found that the enzyme activity was increased 50 to 100% by ACTH and two to five-fold by so-

dium fluoride, an unspecific adenyl cyclase activator. In the study of Shima, the adenyl cyclase activity was found in both fasciculata and reticularis zones of the rat adrenal (244). The activation of the enzyme by ACTH was similar in both zones, but corticoids production was more marked in the zona fasciculata. This observation raised questions about the involvement of cyclic AMP in the regulation of steroidogenesis in zona reticularis. Adenyl cyclase, isolated from an adrenal tumor, was made partially soluble by Pastan et al. by treatment of a lyophilized preparation of the particulate enzyme in a French pressure cell, a Nossal shaker or by sonication in the presence of both a phospholipid and a fluoride (198). This purified adenyl cyclase was shown to be still responsive to ACTH and sodium fluoride activation.

Two groups of workers studied adenyl cyclase activity in different fractions of cellular membranes from adrenal cortex. Both found that adenyl cyclase activity was associated with all particulate fractions, i.e. mitochondrial, microsomal, nuclear and other membranous fractions, and no adenyl cyclase activity was detectable in the soluble fraction. The first group (102) observed that the major fraction of the total enzyme activity was associated with the mitochondrial membrane while the second (231) concluded that the enzyme activity was recovered mainly from the microsomal fraction. The latter group also found that the highest binding activity for cyclic AMP was associated with the microsomes (see also II-B-4-b-dd).

The finding of Ney et al. may be of great endocrinological importance. They first described in 1969 the abnormal regulation of cyclic AMP in adrenocortical carcinoma (190). ACTH failed to increase cyclic AMP and corticosterone formations in glands *in vivo* or in slices *in vitro* unless the tissue was

homogenized and the exogenous ATP added. In 1971, they reported an abnormal responsiveness of adenyl cyclase from cancerous adrenal tissue to various hormones (236). It is known that normal adenyl cyclase of the adrenal cortex responds to ACTH only. In the enzyme preparation from cancerous tissue, cyclic AMP formation was unexpectedly increased by epinephrine, norepinephrine and thyroid-stimulating hormone. This loss of specificity of adenyl cyclase leads to the speculation that the so-called "autonomous" secretion of endocrine tumors may in fact be due to an abnormal stimulation by agents usually inactive in a given gland, regardless of the feedback suppression of the appropriate trophic hormone by the secretion of the tumor.

A few *in vitro* studies on phosphodiesterase activity in adrenal tissue permit some speculation about the possibility of effects of pharmacological agents on this enzyme. In these studies (164, 95), the steroidogenic effect of both ACTH and cyclic AMP was inhibited by imidazole and chlorpromazine. Imidazole was shown to be an activator of the phosphodiesterase (43) while chlorpromazine inhibited the rise of cyclic AMP in other tissues such as brain (195).

bb- effect of exogenous cyclic AMP and nucleotide specificity

The effect of the addition of cyclic AMP was studied in various preparations. The superfusion technique was used by Schulster et al. (239), cell suspension by Sayers et al. (232) and Rivkin et al. (217), monolayer cultures by Kowal (132), adrenal slices by Bieck et al. (15) and simple quarters of glands by Mahaffee and Ney (169). In all of these preparations the addition of cyclic AMP or of ACTH stimulated the steroidogenesis.

Nucleotide specificity was repeatedly confirmed in these experiments. In addition to adenosine cyclic monophosphate, uridine, cytidine, guanosine

and inosine cyclic monophosphates were found to stimulate *in vitro* the production of corticosterone in rat adrenal (169). A similar conclusion was made by Bieck and Rivkin (15, 217). The most effective cyclic nucleotides other than adenosine were those with inosine and guanine bases. The importance of this finding remains to be established since only cyclic AMP and GMP were found to be widely distributed *in vivo* (98). Rivkin et al. demonstrated that the most active nucleotide is a dibutyryl derivative of cyclic AMP and that all cyclic nucleotides showed synergism in presance of 5.0 mM theophylline. Theophylline enhanced the activity of cyclic AMP probably by phosphodiesterase inhibition, while it lowered the activity of ACTH possibly by inhibiting the adenyl cyclase (232).

cc- site of action

We have already discussed the ubiquity of adenyl cyclase within the membrane of adrenocortical cell. The following question may be asked: which step of steroidogenesis is mainly dependent on cyclic AMP mediation since the predilection of specific hydroxylations to particular cell organels is well known (19) ?

It is more probable that cyclic AMP is involved in the mediation of ACTH action in the cleavage of the cholesterol side chain (121). The hydroxylation of cholesterol to 20-hydroxycholesterol and then to 20α, 22-dihydroxycholesterol are the first steps in the biosynthesis of pregnenolone and are rate-limiting reactions in adrenal steroidogenesis (130). In the study of Cohen and Moriwaki on the adrenal desmolase system (46), cyclic AMP added to the incubation medium of guinea pig adrenals produced a three-fold stimulation of the overall synthesis of corticoids. The inhibition of steroidogenesis with

aminoglutethimide (thought to be a competitive inhibitor of 20-hydroxylation) could not however be overcome by addition of cyclic AMP. This led the authors to the conclusion that cyclic AMP does not influence the cholesterol transformation into pregnenolone beyond 20-hydroxylation. Using the monolayer culture of ACTH-responsive cells, Kowal augmented the maximum steroidogenic output, in response to prolonged incubation with ACTH and cyclic AMP (132). Prolonged incubation was associated with a progressive increase in 11β-hydroxylation of endogenously produced steroids. In Kowal's study, cyclic AMP did not stimulate 3β- or 20α-hydroxysteroid dehydrogenases. The author concluded that the action of cyclic AMP increased mitochondrial enzyme activities (11β-hydroxylase) whereas it had no effect on microsomal enzymes (3β- or 20α-hydroxysteroid-dehydrogenase).

Two important observations, the first in rat adrenal and the second in mouse ovary, brought evidence that cyclic AMP enhances the conversion of cholesterol into pregnenolone, and at the same time inhibits the conversion of pregnenolone into progesterone (257, 178). This inhibitory activity was observed in both microsomal and mitochondrial fractions. When Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-3-ketosteroid isomerase were studied individually, it appeared that it was probably the dehydrogenase which was a rate-limiting step. The inhibitory effect of cyclic AMP on the conversion of pregnenolone to progesterone in adrenal subcellular preparations was also primarily related to the action on the dehydrogenase. Another observation of some preferential pathways in adrenal steroidogenesis came from the relatively early work of Péron (205). He showed that ACTH and cyclic AMP stimulate to the same extent the 18-hydroxydesoxycorticosterone and corticosterone production, but that

the stimulation of aldosterone production due to ACTH exceeds that induced by cyclic AMP. These reports lead to the speculation that some internal regulation of different steroid pathways are therefore possible by the intervention of cyclic AMP.

dd- mode of action

Many conflicting reports involving glucose metabolism in the mediation of ACTH action by cyclic AMP have been published, since the time of Haynes hypothesis. Arsenite inhibition of pyruvate dehydrogenase and 2-deoxyglucose inhibition of glycolysis were used to support the idea that pyruvate production was not only an important consequence of the glycolytic activity of cyclic AMP, but that the availability of pyruvate was also necessary for the steroidogenic activity of cyclic AMP (271). In a critical study, Bartová and Birmingham, using different approaches, showed the independence of steroidogenesis on lactic acid production (10) since it was possible to vary both the steroidogenesis and glycolysis in the same or the opposite direction. The authors suggest that increased glycolysis, occurring as well *in vivo*, may facilitate the steroid secretion due to a rise in blood flow caused by its vasodilatator effect. The importance of a specific role of phosphorylase in cyclic AMP mediated steroidogenesis was questionned by Coessens (44). In this study, the phosphorylase activity was measured after ACTH injection *in vivo* and after addition of ACTH or cyclic AMP to beef and rat adrenals *in vitro*. The activation of steroidogenesis was not compulsively accompanied by an increase in the phosphorylase activity on one hand, and an increase in the phosphorylase did not necessarily lead to steroidogenesis on the other hand.

Other workers have suggested the involvement of protein synthesis in the control of steroidogenesis by cyclic AMP. Ferguson was able to abolish the effects of ACTH and cyclic AMP with puromycin, which is known to block the proteosynthesis by removing t-RNA from ribosomes (72). Caution in the interpretation of the data relating to puromycin is necessary as unspecific effects of puromycin, such as a rise of glycogenolysis, were reported (55). Burrow brought conflicting findings by observing a discrepancy between the effects of ACTH and cyclic AMP on proteosynthesis and steroidogenesis in human adrenals; ACTH enhanced proteosynthesis but cyclic AMP did not (31). However, it must be pointed out that the author worked first with tissue which was already hyperplastic (provided from a patient with Cushing disease) and secondly, that he used only one concentration of cyclic AMP, which failed to stimulate the steroidogenesis. In contrast, Grower and Bransome were able to stimulate the steroid biosynthesis in cultures of adrenocortical tumor cells with ACTH and cyclic AMP within 15 to 60 minutes (94). The stimulation of steroid biosynthesis preceeded any significant increase in overall protein synthesis, as verified by [^{3}H]-leucine incorporation. However, when cytosol proteins were fractionated by electrophoresis, rapid changes in the labelling of several protein fractions were evident in less than 30 minutes and no longer evident after 60 minutes. This finding supports the possibility of an involvement of a special protein in induction of ACTH and cyclic AMP regulated steroidogenesis.

Recently, it was demonstrated that the presence of an enzyme, the protein kinase (EC 2.7.1.37), was necessary for the action of cyclic AMP upon many intracellular regulatory systems. This enzyme was isolated from many tissues including the adrenal cortex (146, 85). Gill et al. were able to dissociate

this protein into two subunits one being the receptor and the other the generator (86). The cyclic AMP receptor functions as a repressor of the protein kinase; binding of cyclic AMP to the receptor causes it to dissociate from the generating part of kinase which is then fully activated. This generating subunit of activated protein kinase is able to transfer the gamma phosphate from ATP to the substrate. The major substrates in the adrenal seem to be histones (145) and ribosomes (278). Histone phosphorylation may lead to DNA derepression (151) while ribosome phosphorylation may influence the m-RNA translation process (278). On the basis of the investigations cited in this chapter, a schema has been drawn on the transmission of ACTH informatory effect upon adrenocortical cell (fig. 1).

5 - OTHER HORMONES

a - *Effect of glucagon on heart muscle*

The cardiovascular effects of catecholamines (discussed in part II-B-2-b) and of glucagon were demonstrated in similar type of studies. Glucagon has been shown to produce a positive inotropic effect on normal heart in animals including man (196). This activity is thought to be mediated by an adenyl cyclase-cyclic AMP system. Entman et al. showed that cyclic AMP, in the presence of ATP and Mg^{++}, caused an increase in the rate of accumulation of Ca^{++} in the microsomal fraction of canine myocardium (63). This fraction, consisting primarily of sarcoplasmic reticulum was shown by the same authors to contain adenyl cyclase activity. Glucagon stimulated the Ca^{++} accumulation in a similar manner, but required a longer period than cyclic AMP. This observation fits well into the hypothesis that glucagon must first increase the intracellular level of cyclic AMP before the physiological result becomes ap-

parent. The same group (159) measured recently changes in the adenyl cyclase reactivity to glucagon occurring in heart failure. Increases of adenyl cyclase activity by glucagon in various tissue preparations from normal cats, were as follows: 80% in the left ventricle, 57% in the right ventricle, and 500% in the liver. When tissues from cats with chronic right ventricular failure were studied, the activation of liver adenyl cyclase was identical but no activation was caused by glucagon in the right or the left ventricles. This observation may be of importance in the search for physiopathological mechanism of heart failure.

b - *Effect of thyroid hormone on the heart*

Levey and Epstein demonstrated the stimulating effects of L-thyroxine and L-triiodothyronine on myocardial adenyl cyclase (158). In their preparation of adenyl cyclase, propranolol abolished the activation of the enzyme induced by norepinephrine, but it failed to alter the activation caused by thyroxine. This observation suggests the presence of at least two adenyl cyclases in heart muscle, one sensitive to norepinephrine and glucagon, and the other, to thyroid hormone. The hyperdynamic circulatory state of hyperthyroidism, characterized by high cardiac output and tachycardia, was for a long time attributed to the increased sensitivity of the heart towards catecholamines (274). Several recent observations do not support this hypothesis (156). First, Levey failed to demonstrate any difference in norepinephrine stimulation of adenyl cyclase in the preparation of papillary muscle obtained from euthyroid and hyperthyroid animals (160). Secondly, Klein showed that reserpine does not alter adenyl cyclase activation induced by thyroid hormone in spite of a beneficial circulatory effect of this drug in hyperthyroid patients (128). And

finally, unaltered levels of cyclic AMP have been demonstrated in hearts from hyperthyroid rats (179). Therefore, the observed increase in adrenergic activity in hyperthyroidism must be secondary to a mechanism other than altered receptor sensitivity.

c - *Steroids and catecholamines interaction*

The permissive action of corticosteroids for catecholamine activity has been well established (212). In the absence of these steroids, the threshold for epinephrine and norepinephrine action is raised for every area of their effect. On the other hand, the toxic response to the catecholamines is enhanced in corticoadrenal insufficiency. The pretreatment of patients with ACTH or cortisone results in an increase in pressor response to infused norepinephrine (212).

In trying to elucidate the mode of action of the permissive and interregulating effects of corticosteroids and catecholamines, the system of the second messenger was taken into consideration. The interaction on this level is highly complex and rather opposite effects were observed. Robison et al. found that liver from adrenalectomized rats reacted to epinephrine with a greater than normal response in the level of cyclic AMP and the administration of glucocorticoid suppressed the cyclic AMP response to epinephrine (221). Another example of reduced response of cyclic AMP to catecholamine stimulation came from the work of Weiss and Crayton (282). The administration of estradiol or the endogenous variations of estrogens during proestrus reduced by more than 80% the activation of rat pineal adenyl cyclase to norepinephrine with a lesser effect on base-line activity of adenyl cyclase.

An increase in the activity of the second messenger system by glucocorticoids has been evoked in both metabolic and contractile cardiac functions (106). Exton et al., in their study on the permissive action of glucocorticoids on glycogenolysis, came to the conclusion that the impaired effect of epinephrine in adrenalectomized rats is not due to a defect in the activity of adenyl cyclase (68). Since the glycogenolytic response to low concentrations of cyclic AMP was also impaired, it was postulated that the defect was beyond the formation of cyclic AMP. An attractive explanation was suggested by the work of Senft et al. (241) who observed that an increase in phosphodiesterase activity lead to an acceleration of cyclic AMP destruction in the liver, the muscle and the kidney of adrenalectomized rats. Glucocorticoids decreased phosphodiesterase activity in both *in vitro* and *in vivo*. In the latter experiment this effect may be attributed to a decreased synthesis of the enzyme.

It seems that the effect of steroids may be regulated by catecholamines in a way similar to that in which the effects of catecholamines are or may be modulated by steroid hormones. Szego and Davis found that the β-adrenergic blocking agents precluded the cyclic AMP increase secondary to the administration of estrogens (262).

In summary, the interaction between steroids and catecholamines may be situated on the level of cyclic AMP formation and/or metabolism, depending on the tissue, the animal and the function which is studied. Unfortunately, we were unable to find any literature on the involvement of cyclic AMP in steroids and vasoactive amines interaction on vascular smooth muscle reactivity.

6 - PROSTAGLANDINS

This group of twenty-carbon fatty acids was shown to be ubiquitous and possessing a variety of pharmacological activities. They are not hormones in the sense of an endocrine gland-target organ system, but they appear to serve as local controllers of cellular function (32). Prostaglandins can interact with blocking agents and agonists, potentialize or inhibit smooth muscle contraction and antagonize or mimic the action of hormones. They have important implications in the control of the reproductive or nervous systems and are intimately related to the regulation of lipid and carbohydrate metabolism (13). In several instances, it was reported that their action involved changes in the level of cyclic AMP. The first demonstrated effect, linked with lipolysis, was the fall of the level of cyclic AMP in adipocytes (32). But the most common effect of prostaglandins is to increase the level of cyclic AMP in platelets, thyroid, ovary, heart and kidney medullary tissues (32).

a - *Interaction with catecholamines*

Prostaglandins of the E and F series, applied in subthreshold amounts to a variety of smooth muscles, decreased the effect of catecholamines. On the other hand, the β-blocking agent, propranolol, prevented the miosis induced by PGE_1 in rabbits (13). In a study of venoconstrictor response, Kadowitz observed that the infusion of $PGF_{2\alpha}$ enhanced markedly the responses of the saphenous vein to sympathetic nerve stimulation or injected norepinephrine (115). Wennmalm and Hedqvist found, in isolated rabbit hearts, that PGE_1 counteracted the inotropic response as well as the outflow of norepinephrine following sympathetic nerve stimulation (283). The response to infusion of

exogenous norepinephrine was affected by PGE_1 only slightly and inconsistently. These workers concluded that PGE_1 in low concentrations interferes with the function of the sympathetic neuromuscular system of rabbit heart.

The additive effect of PGE_1 and β-adrenergic activation has been demonstrated on a particulate adenyl cyclase preparation from rabbit ciliary process tissue where PGE_1 and α-adrenergic effects appeared to be antagonistic (273).

Siggins et al. studied interactions between norepinephrine, cyclic AMP and prostaglandins on rat cerebellar Purkinje cells using the microiontophoretic method (247). The depression of the firing rate of single Purkinje cells produced by norepinephrine was mimicked by cyclic AMP and the effect was potentialized by methyl-xanthines. The iontophoresis of prostaglandins E_1 and E_2 (but not $F_{1\alpha}$, $F_{2\alpha}$) known to reduce cyclic AMP in peripheral neuro-effector system, blocked the action of iontophoretically applied norepinephrine in rat cerebellum. These two agents were unable to antagonize the inhibitory effect of cyclic AMP. The authors suggested that PGE_1 and PGE_2 may act as modulators of adrenergic transmission by an effect on adenyl cyclase activity.

b - *Involvement in blood pressure regulation*

The activity of prostaglandins on blood pressure may be a combination of their multiple metabolic effects. A few years ago, Strong et al. purified a vasodepressor lipid from renal medullary tissue and presented the evidence of identity between this lipid and PGE_1 (255). In a later study, the same worker described the effect of prostaglandins E_1, E_2, A_1 and $F_{1\alpha}$ on isolated vascular smooth muscle (254). The isolated aortic and coronary smooth muscle contracted in response to these prostaglandins while the smooth muscle of small ar-

teries from other sites showed a biphasic dose-response relationship. These small arteries became relaxed by prostaglandins in low concentration and contracted further with prostaglandins in higher concentration. Since usually only low concentrations are obtained in *in vivo* conditions, the relaxation of small blood vessels may be the right explanation for the *in vivo* observed vasodilatator effect of prostaglandins.

Prostaglandin E_1 can also influence steroid secretion. Prostaglandins and cyclic AMP duplicated the ACTH effect by increasing steroidogenesis *in vitro* as well as *in vivo* in rats (73). In sodium depleted sheep, Blair-West et al. observed a significant fall of aldosterone secretion rate and an insignificant increase in cortisol and corticosterone secretion rates by PGE_1. In addition Kuehl et al. observed the stimulatory effect of prostaglandins on LH dependent steroidogenesis in mouse ovary by establishing a dose-response relationship between prostaglandins and the formation of cyclic AMP (140). Kinetic studies permitted the same authors to suggest that there is a single luteinizing-hormone-related prostaglandin receptor in mouse ovaries. The activation of this prostaglandin receptor is an essential requirement in the action of LH to stimulate the formation of cyclic AMP and steroidogenesis.

The first human study with prostaglandins was performed in 1959 by Bergström et al. (13). A short infusion of PGE (0.02 to 0.7 µg/kg/min.) produced tachycardia, moderate hypotension and decreased cardiac output. Recently, several infusion studies were performed by the group of Lee (154). In their latest report, PGA_1 was infused intravenously for one hour to six patients with fixed diastolic essential hypertension. Low infusion rates (0.1 to 2.1 µg/kg/min.) were not associated with any change in blood pressure, but resulted in a significant increase in effective renal plasma flow, glomerular fil-

tration rate, urinary flow and sodium and potassium excretion. However, at higher infusion rates (2.1 to 11.2 µg/kg/min.), the blood pressure fell from a mean control of 200/112 mmHg to 140/85 mmHg and the previously increased parameters of the renal function were reduced to the preinfusion level. Therefore, the normotension induced by PGA_1 in hypertensive patients was associated with normal renal blood flow and normal sodium excretion. This extremely interesting observation was unfortunately performed on patients only, without a control experiment in normal subjects. Lee cited a personal communication from Dr Patel who observed that in normotensive subjects low infusion rate (1 µg/kg/min.) of PGA_1 did not cause any change in blood pressure, urinary output or sodium excretion. Finally, patients in the study of Lee received diazepam (Valium^R) 5 to 10 mg intramusculary 15 minutes before the study began and there is an evidence in the literature that many centrally acting drugs affect the concentration of cyclic AMP in the brain (203, 195). Therefore, the modulation of the action of prostaglandins may be to some extent influenced by a premedication.

7 - ELECTROLYTES

a - *Cation involvement in smooth muscle contraction*

In 1954, Tobian and Binion reported an increase in sodium and potassium content and a decrease in the magnesium content in the aortic wall of hypertensive rats (268). Since 1954, there have been many reports on the involvement of electrolytes in the contraction of vascular smooth muscle in vessels of normal and hypertensive animals (250, 251). Friedman proposed the theory that peripheral vascular resistance depends upon the sodium transfer system (77). The contractile response to physiological vasoconstrictor agents is

characterized by a shift of sodium into, and of potassium out of, the "cellular" phase of the arterial tissue (123). More recent and detailed studies of Friedman and Friedman provide evidence implicating the ion-exchange capacity of the matrix in the genesis of s sustained increase in peripheral vascular resistance (78).

Probably all significant physiological changes in vascular reactivity are accompanied by an alteration in the concentration of the calcium ion in the environment of the myofilament (123). We have already discussed the relationship between the accumulation of calcium in smooth muscle and the activation of adenyl cyclase by norepinephrine, as was reported by Epstein et al. (66). Seidel and Bohr showed the existence of a relationship between norepinephrine-induced contraction and reduced calcium efflux, as well as between isoproterenol-induced relaxation and decreased calcium influx (240).

It was shown that the chemical changes present in hypertensive vascular smooth muscle are similar, regardless of the cause of the hypertension (18). These changes were taken into account in the study of Bohr and Sitrin for the explanation of increased excitability to norepinephrine, KCl and $CaCl_2$ in vascular muscle from hypertensive animals.

Somlyo et al. showed that the potassium-dependent β-adrenergic hyperpolarization may be mediated by cyclic AMP (249). In their study, db-cyclic AMP and theophylline were able to hyperpolarize the smooth muscle of rabbit pulmonary artery, only in the presence of a low potassium concentration. This dependence of activity on low potassium concentration was similar for cyclic AMP and isoproterenol.

Disturbances of vascular reactivity to angiotensin infusion were observed in different pathological stages, such as hyper- and hypoaldosteronism, cyclic

edema and hypertension, all related to alteration of sodium homeostasis (138, 139, 120). Heistad et al. studied the influence of sodium concentration on vascular reactivity *in vivo* in man (104). The vasoconstrictor response of norepinephrine and angiotensin was reduced at low and increased at high intravascular sodium concentration. The reflex vasoconstriction, activated by lower body negative pressure, was similarly affected by changes in sodium concentration.

b - *Intake and excretion of cations*

Many authors have studied the relationship between disturbances in sodium metabolism in human or experimental hypertension. Genest summarized these as follows (83):

1. *Greater and more rapid rejection of salt loads. Blockage of this effect by propranolol.*
2. *Higher incidence of hypertension in population with high salt intake.*
3. *Increased arterial sodium content in various types of hypertensive states and preceeding the rise of blood pressure in DOCA hypertension.*
4. *Antihypertensive effectiveness of:*
   (a) *Severe sodium restriction (or rice-fruit diet) in 30 to 50% of patients with essential hypertension.*
   (b) *Natriuretic agents and spironolactone.*
5. *Natriuretic effects of angiotensin despite aldosterone stimulation.*
6. *Potentiation of hypertensive effects of steroids by administration of sodium.*
7. *Production of hypertension in salt-sensitive strain of rats or by high salt intake in humans.*

8. *Prevention of toxemia of pregnancy by low sodium intake and/or thiazide drugs.*
9. *Pressor response to cross-transfusion of blood from a renal hypertensive rabbit to a high salt-fed rabbit.*

In another study, the restriction of sodium intake lowered norepinephrine and sodium contents in all vascular tissues, with the exception of the renal artery that showed no change (100). The major sodium regulating hormone, aldosterone, was shown to have a decreased metabolic clearance rate in patients with benign essential hypertension (191). This fact could account for the elevated plasma concentration of aldosterone even when the secretion rate is normal or low. In section II-B-3-c, we have already discussed a possible relationship between cyclic AMP and the action of aldosterone.

Thorn was first, in 1938, to suggest an effect of sex hormones on the renal excretion of electrolytes (267). The groups of Landau and subsequently Laidlaw established that progesterone can counteract by competitive inhibition the effects of aldosterone whether from endogenous sources or exogenously administrated (149, 150, 147). Laidlaw demonstrated that the natriuretic effect of progesterone may be present only initially since after a period of time, progesterone administration promotes a rise in aldosterone excretion and secretion rates in young normal subjects (147).

Renal regulation of water excretion, including both control of urine concentration and dilution by the action of ADH, are well established (127, 108). The activity of ADH is mediated through adenyl cyclase-cyclic AMP system (194, 113, 12) as well as the natriuretic effect of vasopressin (171, 183). In addition, Handler et al. and Watlington (96, 276) studied the action of adrenergic agents on the effect of ADH. The former group observed that the water per-

meability response to toad urinary bladder to ADH was inhibited by catecholamines without however inhibiting the response to cyclic AMP. This inhibitory effect was counteracted by α-blocking agent but not by β-blocking agent. From the study of Watlington it was concluded that the inhibitory effect of α-adrenergic stimulation on the ADH mediated sodium transport is due to a decreased formation of cyclic AMP. Gill and Casper demonstrated in dogs that the β-adrenergic stimulation by isoproterenol depressed the proximal tubular sodium reabsorption, possibly through mediation of the adenyl cyclase system (87).

The excretion of calcium after sodium-load was enhanced in hypertensive subjects in the study of Ackerman (2). The regulation of calcium excretion by parathormone is also mediated through cyclic AMP (39, 7). In relation to the effect of parathormone and ADH on adenyl cyclase in the kidney, it is important to note that the parathormone has a striking effect in the cortical preparation while vasopressin produces a similar effect in the medullary preparation (38).

c - *Cations and metabolism of cyclic AMP*

The efflux of potassium ions is the first event observed in response to epinephrine, glucagon or cyclic AMP in the liver; it comes even before the liberation of glucose (50). This interesting finding was never elucidated (221). The increase in potassium/sodium ratio was shown to stimulate the effects of luteinizing hormone and of cyclic AMP on progesterone synthesis in corpus luteum of rat (105).

Bivalent cations are of importance for the metabolism of cyclic AMP. The magnesium or manganese cations are required for the transformation of ATP to cyclic AMP by adenyl cyclase (211). The effect of calcium ion on adenyl cyclase activity is more complex. In the cardiac tissue, the removal of Ca^{++} from in-

cubation media caused a significant elevation in cyclic AMP and an excess of Ca^{++} decreased the concentration of the cyclic nucleotide (189). The inhibitory effect of Ca^{++} on adenyl cyclase may be explained by a competition with Mg^{++}, however a possible effect on phosphodiesterase must also be considered (see below). In the adipose cells, the presence of Ca^{++} is required for the formation of cyclic AMP and lipolytic effects of ACTH and of β-lipolytic hormone (144, 165). On the contrary, the effect of norepinephrine was independent of Ca^{++} and the presence of this cation lowered somewhat the basal activity of adenyl cyclase (144). Carchman et al. observed that in perfused adrenals, the presence of Ca^{++} in the medium was required for the steroidogenesis but not for the formation of cyclic AMP (35), the production of which was even enhanced in the absence of calcium in perfusion medium.

Cyclic AMP hydrolysis by phosphodiesterase is highly dependent on the ionic environment. A partially purified preparation of brain phosphodiesterase contained Ca^{++} and Zn^{++}(41, 42). The enzyme activity was stimulated by Mg^{++} and Cu^{++}, whereas Ca^{++}, Mn^{++}, Co^{++} and Zn^{++} were stimulatory at low concentrations and inhibitory at high concentrations. EDTA exerted a 50% inhibition of phosphodiesterase at a concentration of 7 μM which was completely abolished by magnesium.

Rasmussen advanced the hypothesis that the activation of adenyl cyclase, leading to the formation of cyclic AMP and the activation of protein kinase, consequently stimulates a membrane bound system (214). The role of this system is to increase the influx of calcium into the cell. The author ascribes to the mobilization of calcium the responsibility for the major part of intracellular, hormonally regulated events such as the action of parathormone, the secretory mechanisms, and the release of synaptic transmitters.

The involvement of cyclic AMP, in some responses of hormones participating in the regulation of blood pressure, is summarized in table 1.

C - *IN VIVO* STUDY OF CYCLIC AMP

1 - METABOLIC STUDIES

Cyclic AMP was first isolated from human urine by Butcher and Sutherland in 1962 (33). More recently, many authors, using different techniques, have been able to measure cyclic AMP in human urine with similar results (refer to table 2). It has been established by Price et al. that the only organophosphorus compounds present in the non-lipid fraction of rat urine are cyclic AMP and cyclic GMP (209). Cyclic GMP represented about 30% of (^{32}P) in this study involving the incorporation of phosphorus.

In the study of Chase and Aurbach, the injection of parathyroid hormone drastically increased the excretion of cyclic AMP in the urine of rat and man (37). The excretion of injected radioactive cyclic AMP was unaffected by parathormone while the kidney adenyl cyclase was activated by this hormone. These two facts led the authors to the conclusion that urinary cyclic AMP originates from the kidneys (38). They attributed to parathormone the major controlling activity of cyclic AMP excretion, based on the observation of the suppression of cyclic AMP excretion by the infusion of Ca^{++}. It was later demonstrated by the same authors that only a fraction of the total cyclic AMP excreted in human subjects is controlled by the parathormone (39). The inhibition of parathormone secretion, by calcium infusion into man, caused only a slight fall in urinary excretion of cyclic AMP. Broadus et al. measured concomitantly the urinary excretion and the plasmatic level of cyclic AMP and the inulin clearance in man (23). They established that between one half to two

TABLE 1

Involvement of cyclic AMP in some responses of hormones regulating blood pressure

HORMONE	RESPONSE	EFFECT ON cAMP CONTENT	REFERENCES
Catecholamines	Positive inotropic response (heart)	↑	141,157,66,54,221
	Ca^{++} accumulation in sarcosplamic reticulum (heart)	↑	245, 66
	Smooth muscle contraction (aorta)	↓ ↑	272,235
	Cell polarization (nervous tissue)	↑	176,248
	Renin release (kidney tissue)	?	185,264,287,99
Angiotensin	Smooth muscle contraction (aorta)	↓	272
	Steroidogenesis (adrenal cortex)	?	119
Aldosterone	Sodium transport (kidney tubule)	?	125
ACTH	Steroidogenesis (adrenal cortex)	↑	101,122,198,102,231,164 190
	Renin release (kidney tissue)	?	99
Glucagon	Positive inotropic response (heart)	↑	66,63
	Ca^{++} accumulation in sarcosplasmic reticulum (heart)	↑	66
Prostaglandins	Diminution of catecholamine effect (smooth muscle, heart)	↓	13,283,248
	Steroidogenesis	↑	73,16,140

TABLE 2

Normal plasma and urinary level of cyclic AMP

AUTHOR	SPECIES	PLASMA nM	URINE		
			µM	µmol/day	µmol/g of creatinine
Butcher and Sutherland (1962)	Man			2-7	
Price et al. (1967)	Rat			0.050-0.058*	
Aurbach et al. (1970)	Man			1.4-6.0	2.4-5.4
Davis et al. (1969)	Man			4.4;6.5	
Goldberg et al. (1969)	Man			2.3;3.4	
Hardman et al. (1969)	Rat			0.059-0.066*	
Kaminsky et al. (1969)	Man	10-20			
Johnson et al. (1970)	Man		0.52-0.66		
Taylor et al. (1970)	Man			3.54‡0.17	
Broadus et al. (1970)	Man	12.5-24.8		3.7-7.2	1.6-3.1
Kaminsky et al. (1970)	Man	12-25			1.5-5
Present study (1972)	Man				1.1-5.5

* per 100mg of body weight

thirds of cyclic AMP excreted in the urine is derived from the plasma by glomerular filtration, the remainder being produced by the kidney itself. The renal production of cyclic AMP is partly under the control of parathormone. Renal production can be suppressed by the infusion of calcium and stimulated by the infusion of EDTA, a calcium-chelating agent (118).

There are conflicting results concerning the effect of ADH on cyclic AMP excretion. An increase in cyclic AMP excretion upon ADH infusion was reported by Davis (51) and Taylor (263), however no change was observed by Chase (39) and Kaminsky (117). Another hormone having a pronounced effect on cyclic AMP is glucagon. Its injection into rat (97) and human (24) produced a several-fold increase in plasmatic level and urinary excretion. Administration of epinephrine had a similar although less marked effect. Kaminsky et al. demonstrated an increase of plasma level of cyclic AMP by β-stimulation with isoproterenol (116). In contrast, α-stimulation was characterized by a rise of plasmatic cyclic GMP. These workers noted that the β-adrenergic stimulation seemed to diminish the nephrogenous cyclic AMP. It means that the actually observed increase in cyclic AMP in urine was less than calculated from plasma cyclic AMP and inulin clearance.

Hardman et al. demonstrated in rat that the urinary excretion of cyclic GMP is more dependent on the presence of hypophysis than that of cyclic AMP (97). The lowering effect of hypophysectomy on the urinary excretion of cyclic nucleotides was reversed by hydrocortisone in the case of cyclic AMP, but its combination with thyroxine was necessary in the case of cyclic GMP. In addition, the adrenalectomy decreased only cyclic GMP and thyroparathyroidectomy only cyclic AMP excretions. Taylor observed an increase in cyclic AMP excretion at the time of ovulation and also during normal pregnancy in human (263).

Reports concerning disturbances in cyclic AMP in pathology of the human central nervous system were introduced by the findings of Abdulla et al. (1), who reported a decrease of cyclic AMP excretion in depressive states and its increase in mania. These findings have been contested by some workers (222), and seem to be confirmed by others (213, 200, 201).

In 1970, Broadus et al. performed an extensive study on kinetic parameters and renal clearances of cyclic AMP and cyclic GMP in man, using tritium-labelled cyclic nucleotides (23). Both cyclic nucleotides were cleared from plasma by glomerular filtration. The kidney contributed by about one-third to the total amount of the cyclic AMP excreted, while plasma was the only source of cyclic GMP excreted. Plasma production rates were also established. Approximately 85% of the elimination of the cyclic nucleotides was due to the extra-renal clearance.

2 - EFFECT OF EXOGENOUS CYCLIC AMP

The hormone-like effect of exogenously administrated cyclic AMP is required to fulfill one of Sutherland's criteria for acceptance of cyclic AMP mediation of hormone action (261). In 1962, Posternak first demonstrated the hyperglycemic effect of exogenously administered db-cyclic AMP in dog (221).

Cyclic AMP is relatively inert substance when applied to intact cells or when injected into intact animals (221). Pharmacological doses must be used for the demonstration of its activity. Two reasons have been enunciated, one is the difficulty of passage of cyclic AMP into the cell and the other, its rapid destruction by ubiquitous phosphodiesterase. The relatively high activity of this cyclic AMP catabolizing enzyme was also observed in plasma by Patterson et al. (199). Cyclic AMP destruction in plasma explains probably

in part a frequent adenosine-like effect of exogenously administered cyclic AMP. The preparation of analog and derivatives of cyclic AMP, mainly of mono- and dibutyryl (N^6 and $O^{2'}$) derivatives, was undertaken in order to solve the above mentioned problems (69). Blecher demonstrated that the presence of substituent groups in N^6 and $O^{2'}$ positions of cyclic AMP hinder sterically the effect of cyclic nucleotide phosphodiesterase (17).

Dibutyryl derivative of cyclic AMP was used also in human study by Levine (163). He demonstrated the chronotropic and hyperglycemic effects in human volunteers by intravenous administration of 0.3 to 0.5 mg/kg/min. of db-cyclic AMP. In another study by the same author, the administration of the parent compound required much larger doses to demonstrate the activity and was accompanied by many side effects (161, 162).

D - QUANTITATIVE DETERMINATION OF CYCLIC AMP

The first assay for the determination of minute amounts of cyclic AMP present in biological tissues and fluids was described in 1958, two years after the discovery of the nucleotide by Rall and Sutherland (210). This assay is based upon the ability of cyclic AMP to accelerate the conversion rate of the inactive liver phosphorylase to its active form. The procedure requires preparation of inactive liver phosphorylase, phosphorylase kinase, and cyclic nucleotide phosphodiesterase. This assay, with its subsequent modifications (221), is still used today at Vanderbilt University and elsewhere, and remains an excellent tool in skilled hands. However, its complexity precludes a widespread utilization. Its limitation is seriously accentuated by the quantitative variability of results assayed under identical conditions. Without ques-

tion, the use of this assay permitted many of today classic discoveries of hormonal action on cyclic AMP and on metabolical fates of the nucleotide.

Since the early sixties, a large variety of methods for the quantitative determination of cyclic AMP have been developed and some of them should be briefly characterized. In 1964, Breckenridge proposed an assay based on a series of enzymatic reactions which convert first cyclic AMP to 5'-AMP (by phosphodiesterase), then to ADP (by myokinase, using a small amount of ATP as a trigger), and finally to ATP (by pyruvate kinase, using the phosphoenol-pyruvate as substrate) (21). The so-formed ATP is further estimated by means of NADPH generation, reflecting the original concentration of cyclic AMP.

The same approach for the generation of ATP from cyclic AMP was adopted by Aurbach and Houston in 1968 (6). In their assay, the generated ATP is estimated by labelling of gamma phosphate of the nucleotide by enzymatic exchange reaction with radioactive phosphate. Details of this method are described in part III of the present thesis. The ATP generating reaction is also used in the assay described by Goldberg et al. (90) and Johnson et al. (114), their methods differing only in the mode of ATP estimation. Brooker reported an assay of an enzymatic radioisotope dilution, based on specific destruction of cyclic AMP by cyclic nucleotide phosphodiesterase to 5'-AMP and subsequent cobra venom induced release of phosphate from 5'-AMP (27a).

Two recent methods have received widespread attention: the radioimmunoassay of Steiner et al. (252) and the protein-binding method of Gilman (88). Both are described in detail in part III of this thesis. A comparative table (table 3) presents a summary of basic characteristics of some assays of cyclic AMP.

TABLE 3

Characteristics of the leading assays for cyclic AMP

ASSAY	PRINCIPLE	RANGE OF STANDARD CURVE	LOWEST SENSITIVITY	SAMPLE PURIFICATION	NOTE
Rall-Sutherland (1958) (210)	phosphorylase activation	$1\text{-}30\times10^{-8}$ M		~ required	laborious preparations
Breckenridge (1964) (21)	enzyme conversion (and NADPH generation)	$2\text{-}24\times10^{-8}$ M		required	
Aurbach-Houston* (1968) (6)	enzyme conversion (and P^{32} exchange)	$0.6\text{-}25\times10^{-2}$ M	6×10^{-12} M	required	day-to-day variation (enzyme inhibitors?)
Brooker et al. (1968) (27a)	radioisotope dilution	ten fold range	20×10^{-12} M	required	flat curve
Goldberg et al. (1969) (90)	enzyme conversion (and NADPH generation)	$1.25\text{-}7.5\times10^{-8}$ M	0.01×10^{-12} M	required	less sensitivity found in other laboratories
Steiner et al.* (1969) (252)	radioimmunoassay	$2\text{-}200\times10^{-9}$ M	$1\text{-}2\times10^{-12}$ M	unnecessary	antibody preparations
Johnson et al. (1970) (114)	enzyme conversion (and luciferase system)	$7.2\text{-}720\times10^{-9}$ M		required	special instrumentation
Gilman* (1970) (88)	protein binding (muscle protein kinase)	variety of curves, as required.	$0.05\text{-}0.1\times10^{-12}$ M	unnecessary	simple and versatile assay
Konijn (1970) (129)	myxamoebe aggregation		10^{-12} g		bioassay
Ebadi (1970) (58)	enzymatic conversion (and luciferase system)	$0.01\text{-}10\times10^{-9}$ M	5×10^{-14} M	required	
Walton (1970) (277)	protein binding (adrenal protein)	$5\text{-}40\times10^{-8}$ M	20×10^{-12} M	unnecessary	low sensitivity
Wastila (1971) (279)	protein kinase activation	$1\text{-}7\times10^{-8}$ M	5×10^{-13} M	unnecessary	

* Used in present study. Refer to part III of this thesis.

III - METHODS

A - HUMAN STUDY

1 - CHOICE OF NORMAL SUBJECTS

Healthy volunteer subjects were mostly university students. Excluded from this study were subjects with history of presence of familial hypertension in parents or siblings, mental, cardiac, hepatic or renal diseases, as well as subjects under continuous medication (including oral contraceptives). In all subjects, complete physical examination revealed no abnormality. Blood pressure (three measurements in upright and recumbent positions) was always below 135/90 mmHg. Basic laboratory data-hemogram, white blood cells count, creatinine, blood urea nitrogen, glycemia, serum calcium, potassium and sodium, total proteins and cholesterol, microscopic and bacteriologic examination of urine were always within normal limits in all the subjects.

One subject (J.P.P.) was originally considered as normal on the basis of the above criteria; it was noticed however that his mother had high blood pressure in late pregnancy. This subject was later eliminated from the group of normals, because his blood pressure measured three times a day during the time of the investigation was on several occasions above normal (>150/90). He complained of anxiety and emotional palpitations. His physical examination revealed dermographism and he was included into the group of patients with labile hyperkinetic hypertension. At the time of writing this thesis (i.e. 2 1/2 years after the initial examination), this subject has sustained high blood pressure with levels ranging between 160 to 170/90 to 100 mmHg as recorded during monthly visits, with a pulse rate exceeding 120 beats/min. on assuming

upright posture. The beta-blocking therapy has been recently instituted (Inderal^{R}, 40 mg q.i.d.) with a resulting normalization of blood pressure, pulse rate and subjective relief of anxiety.

2 - GROUPING OF PATIENTS

Patients included in this study were divided into two groups. The division was based on subjective complaints, physical examination, renal functional studies, intravenous rapid sequence pyelography, isotopic renogram and scintiphotography, and in large majority of patients, on the basis of renal arteriography.

The first group consists of 24 patients with labile benign essential hypertension. They are characterized by hyperkinetic circulation, lowering of blood pressure at bed rest on hospitalization, an exaggerated pulse rate in response to upright posture, dermographism, dyspepsia, anomalies in blood glucose tolerance test, anxiety and/or depression (for a more detailed definition, refer part II-A-2). The second group comprises 13 patients with stable benign essential hypertension. Patients with an accelerated or malignant phase of hypertension, as evaluated on the basis of blood pressure and fundi examination, and patients with renal artery stenosis were excluded from the present study. Female patients receiving oral contraceptives were also not studied.

3 - PROTOCOL OF INVESTIGATION

a - *Conditions*

The study involving control subjects was performed in the Clinical Research Institute of Montreal, while that on patients was conducted in the metabolic

unit of Hotel-Dieu Hospital of Montreal, with the help of the same nursing staff in both instances. All subjects during the period of the study received an identical diet, controlled by professional dietitian , containing 135 mEq of sodium and 90 mEq of potassium per day. Smoking and beverages containing xanthines were forbidden for at least twelve hours prior to urine collection. All subjects adhered to a standard liquid intake consisting of: 600 ml of liquid between 18:00 hours and midnight the evening preceeding the test and 125 ml per hour during the test including the breakfast liquid; this meal was administered before the beginning of the collection.

b - *Study of the effect of body position*

This study was performed on the fourth and fifth day while on controlled diet. Subjects were in recumbent position after 23:00 hours since the preceeding evening. They first voided (miction discharged) at 8:30 hours in recumbent position and then the urine was collected between 8:30 hours and 12:30 hours. One day the collection was taken with the subject recumbent and at rest, and the other day with the subject up and walking leisurely during the entire four hour period with only short intervals of sitting. The sequence of positions was randomized previously for all subjects. Urine was collected on ice and immediately frozen at $-90^{o}C$ to await the determination of cyclic AMP. Blood was withdrawn at 12:30 into heparinized tubes precooled to $4^{o}C$, then immediately centrifuged at 8000 x g for ten minutes in the refregirated centrifuge. The separated plasma was transfered to vials precooled to $-40^{o}C$ and kept at $-90^{o}C$ until the determination of cyclic AMP.

c - *Study of the effect of isoproterenol infusion*

This study was performed on the sixth day on the above standard diet. The same condition of bed rest, diet and hydration were applied as in the study of the effect of body positions. The infusion of isoproterenol (Isuprel^{R}) started after the breakfast and miction at 8:30 hours. The isoproterenol was added to 240 ml of 5% glucose and infused by maintaining a rate of 20 ng/kg of body weight/min. during four hours, unless indicated differently (refer results).

d - *Study of the effect of propranolol administration*

This study was performed on some subjects included in paragraph (a). The conditions of bed rest, standard diet and hydration were as described before. The experimental design consisted of two periods of five days, with a study of the effect of body position on the fourth and fifth, and the ninth and tenth days respectively. For the entire period of ten days, the subjects received continuously either of the two medications, a placebo or a β-blocking agent in alternate periods. The β-blocking agent was propranolol (Inderal^{R}) in form of pills, identical in color and size with its placebo, both kindly supplied by Dr M.R. Dufresne from Ayerst Laboratories. The propranolol in dosage of 40 mg was administered q.i.d. and its placebo in the same intervals. The sequence of administration of the drug or placebo, as well as the sequence of positions, were randomized and conducted in a double-blind study.

B - MEASUREMENT OF CYCLIC AMP CONTENT

1 - ENZYMATIC METHOD

The enzymatic method applied in our laboratory, at the beginning of this study, was that described by Aurbach and Houston in 1968 (6).

a - *Material*

All chemicals were of Analytical Reagent Grade. All the water used was glass bidistilled. Carrier-free radioactive phosphate in 0.1 N HCl was purchased from New England Nuclear, Chicago. Both the absence of carrier and the presence of acid were essential; the presence of inorganic phosphate lowers the specific activity and results in a flat standard curve while the acid is necessary to avoid the formation of polyphosphate which in turn impairs the precipitation of the phosphate (256).

Cyclic AMP phosphodiesterase (EC 3.1.4.1) was originally prepared in our laboratory by the method described by Butcher and Sutherland (33) and later purchased from Sigma Co., St.Louis. Pyruvate kinase (EC 2.7.1.40, 425 units/mg of protein), 3-phosphoglyceric phosphokinase (EC 2.7.2.3, 1600 units/mg of protein) and myokinase (EC 2.7.4.3, 640 units/mg of protein) were also purchased from Sigma Co. These enzymes, which are supplied as an ammonium sulfate suspension, were originally dialyzed against 4000 ml of 10^{-3} M Tris-EDTA, pH 7.5 as is recommended by Aurbach and Houston. The only disadvantage of this procedure, necessitating quick freezing and storage in microtubes after the dialysis, is that it is time and material consumming. On suggestion of R.A. Johnson (1969, personal communication), an alternative method of purification

was tried. This purification has two objectives: 1) elimination of ammonium sulfate; 2) elimination of contaminating 5'-AMP from enzymes which raises the blank of the standard curve. The procedure requires: a) centrifugation in the cold at 20 000 x g; b) separation of the ammonium sulfate which remains in the supernatant, followed by one washing with equal volume of a Tris-HCl buffer at pH 7.3; c) resuspension in the same volume of Tris-HCl buffer at pH 7.3; d) passage through a column of Dowex AG -2-X8 (100-200 mesh, Cl^- form), purchased from Bio-Rad, Richmond. The column was 6.5 cm high in a Pasteur pipette, and the elution was performed with the same buffer. The recovery of enzyme protein was measured using the method of Lowry et al. (168). In our experience, the maximum amount of protein representing nearly 100% of its original quantity, was recovered between 0.75-1.5 ml of the eluate. The results of the recovery of protein are shown in table 4. This procedure, proved to be effective in reducing the experimental blank value, suffered from frequent and uncontrollable loss of the enzyme activity. For this reason, in the routine work, only the first three steps eliminating the ammonium sulfate were retained. Immediately before use, the required quantity of each enzymes was mixed and purified as described, permitting to avoid congelation of micro-amounts.

^3H -Cyclic AMP (14.2 to 16.3 Ci/mmol) was obtained from New England Nuclear. The radiochemical purity of this nucleotide was checked by thin-layer chromatography in n-heptane-acetone-isopropanol-0.03 M NH_4HCO_3, pH 8.3 (4:2:8:0.5) (153). All batches used were found to be more than 99% pure. Typical results of chromatography are shown in fig. 2.

Dowex AG 50W-X4, hydrogen form (100-200 mesh) was purchased from Bio-Rad, Richmond. Dowex 50 was extensively washed with bidistilled water before use in order to remove fine particles.

TABLE 4

Purification of enzyme by Dowex 2.

Experiment I

Fraction no.	Tris HCl pH 7.3 elution (in ml)	Reading at 640 mμ	μg of protein	% of recovery
STANDARD	0.5 (of enzyme*)	0.267	130	100
1	0.5	0.000	0	0.0
2	0.5	0.063	28	21.5
3	0.5	0.181	90	69.2
4	0.5	0.008	4	3.0

Experiment II

STANDARD	0.5*	0.254	125	100
1	0.75	0.006	5	4.0
2	0.5	0.170	85	68.0
3	0.5	0.078	38	30.4
4	0.5	0.000	0	0.0

* Enzyme used in these experiments was glyceraldehyde-phosphate dehydrogenase

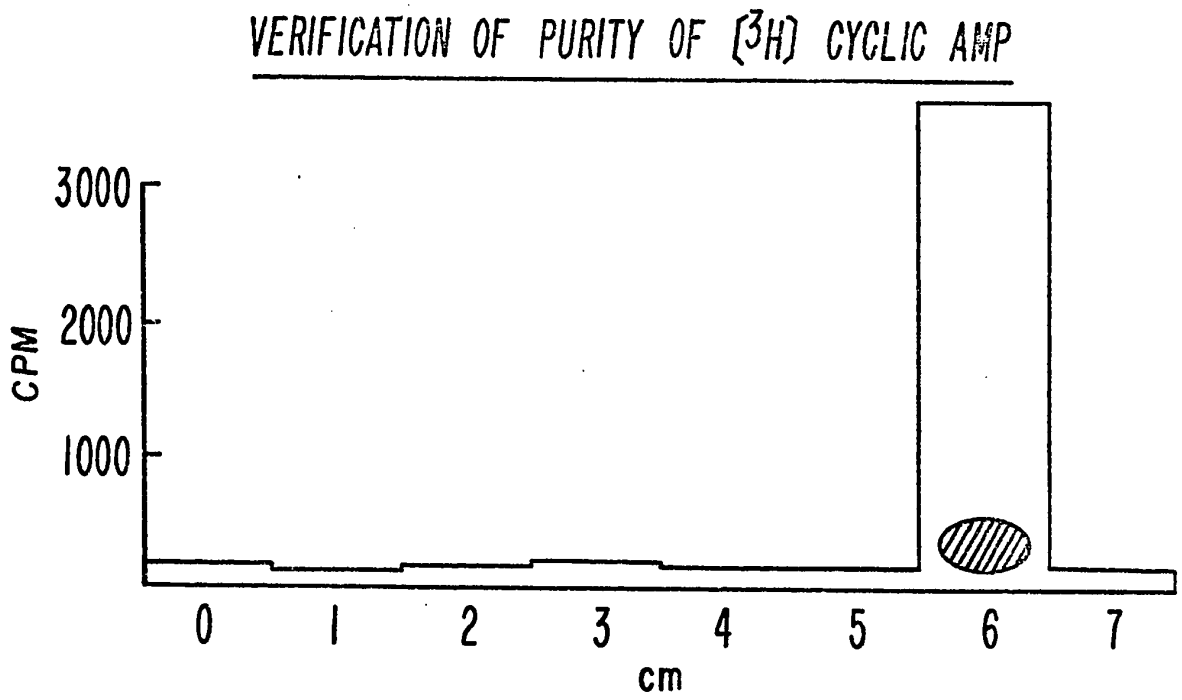


Fig. 2 Thin-layer chromatography of [^{3}H]-cyclic AMP (16.3 Ci/mmol) on silica gel glass microfiber sheet (Gelman Instrument Co., Ann Arbor, Mich.). Solvent was n-heptane-acetone-isopropanol -0.03 M NH_4HCO_3, pH 8.3 (4:2:8:0.5) and the running time 24 minutes. Unlabelled cyclic AMP (1 µg/µl) migrated at the same rate as the labelled material. It was visualized under UV light and the radioactivity of the spot determined by cutting the UV absorbing area into 1 cm strips and counting in a liquid scintillation counter. Of a total of 3580 CPM applied in a single spot, 3540 CPM were recovered following chromatography and elution.

b - *Preparation of phosphodiesterase*

The enzyme purification procedure used was as described by Butcher and Sutherland in 1962 (33), but it was conducted up to heat denaturation step only. Imidazole, grade III, was purchased from Sigma Co., St.Louis. Diethylaminoethyl (DEAE) cellulose was purchased from Eastman Organic Chemical,New York.

It was readied for use by suspending 15 g in one liter of bidistilled water and decanting the finer suspended particles five times. The slurry was first adjusted to approximately 0.02 M of imidazole buffer at pH 7.0 and 0.08 M KCl and was then titrated to pH 7.0 by glass electrode with 1.0 N HCl.

Step I: *Preparation of the extract.* Three fresh beef hearts were obtained from a slaughterhouse and kept on ice until preparation commenced (about 30 minutes later). After clearing of fat and connective tissues, ventricular muscle was cut into strips and washed in 0.33 M sucrose. The strips were homogenized with two volumes of sucrose in a Waring Commercial Blender at a high speed and the homogenate centrifuged at 2000 x g for 45 minutes in the cold. Supernatant fluid was decanted through glass wool prewashed in 0.33 M sucrose.

Step II: *Ammonium sulfate fractionations.* The extracts, kept on ice, were adjusted to half of the saturation concentration with solid ammonium sulfate. Neutrality was maintained by adding 1 N KOH as required. After standing for 30 minutes the extracts were centrifuged in the cold at 8000 x g for 40 minutes, the precipitate was separated and taken up in 15 % of the initial volume of 1 mM $MgSO_4$ and 1 mM imidazole, at pH 7.5 prepared in cold glass bidistilled water. Neutralized saturated ammonium sulfate was added to give a final concentration of 0.45 of the saturation point. After 30 minutes of stirring, the precipitate was collected by centrifugation at 8000 x g for 40 minutes and finally taken up with 5% of the initial volume using as before 1 mM $MgSO_4$ and 1 mM imidazole.

Step III: *Dialysis and freezing of 0.45 ammonium sulfate fraction.* The resulting solution was dialyzed in the cold in Najac casing, 36/32in in diameter (Visking Corp.) against 20 volumes of the same as above solution of magnesium sulfate and imidazole. Following the dialysis, the solution containing heavy flocculent material was frozen at -20°C until the next day. Upon thawing, the floc was collected by centrifugation for 40 minutes at 20 000 x g with the supernatant being conserved and refrozen at -20°C until the next day.

Step IV: *Fractionation in DEAE cellulose.* On the third day, DEAE cellulose, prepared as describe above, was packed up into a chromatographic column under N_2 pressure to give a flow rate of between 0.5 and 0.8 ml/min./cm^2 of column area. The final cellulose bed was 8 x 0.9 cm. From this point on, all operations were carried out in a cold room at 4°C. The phosphodiesterase preparation was adjusted to pH 7.0 in 0.01 M imidazole and 0.08 M KCl. The column was first washed with several bed volumes of cold 0.01 M imidazole and 0.08 M KCl, before the application of the enzyme preparation. Following the seeping in of the sample into the column, two bed volumes of 0.01 M imidazole, pH 7.0 and 0.08 M KCl were passed through. The elution was accomplished with 0.02 M imidazole, pH 6.0 and 0.4 M KCl. The first two bed volumes of the eluate were immediately dialyzed in the cold against 20 volumes of 1 mM imidazole, pH 7.5 and 1 mM $MgSO_4$ with three outside bath changes during 12 hours. After dialysis, the enzyme was frozen in micropipettes in a mixture of acetone and dry ice and kept at -90°C until used.

This preparation of phosphodiesterase was used during the first year of our study, until that of Sigma Co. became commercially available. The activ-

ity of our preparation or of the purchased enzyme was not measured exactly, but only estimated by the chromatography of the product of incubation. This was performed with 1 to 5 µl of the enzyme preparation diluted in 10 µl of Tris-imidazole 0.04 M buffer, pH 7.8 followed by an incubation of phosphodiesterase with tritiated cyclic AMP and 100 µµmol of unlabelled cyclic AMP suspended in Tris 5 mM, pH 8.0 and 1.8 mM $MgCl_2$. A parallel incubation was performed with the omission of phosphodiesterase. The experiment was conducted at 38°C for 20 minutes and stopped by boiling. Aliquots of 10 µl of each incubation, with and without phosphodiesterase, were chromatographed. As evident from fig. 3, cyclic AMP standard or cyclic AMP incubated without phosphodiesterase migrates up to 6 cm, while that incubated with phosphodiesterase remains on the starting line as does the standard of 5'-AMP.

c - *Extraction of cyclic AMP from urine*

Cyclic AMP and cyclic GMP are the only nucleotides present in urine (5). It seems therefore unnecessary to purify the urine for an enzymatic assay, such as that of Aurbach and Houston, when the only substances which could possibly interfere - 5'-AMP and ATP - are not present in urine. These authors, however, recommend to purify the urine without providing any explanation. It was observed, in our study, that it is of primary importance to eliminate the inorganic phosphate from urine to avoid any lowering of specific activity of [^{32}P] tracer necessary for the radioactive phosphate exchange reaction.

An extraction procedure of cyclic AMP from urine was performed, using a modification of the original procedure of Aurbach and Houston. 1.25 ml of urine were quickly thawed and about 40 000 DPM of [^{3}H]-cyclic AMP added; 0.25 ml was separated for counting of the recovery of the standard. The rest of

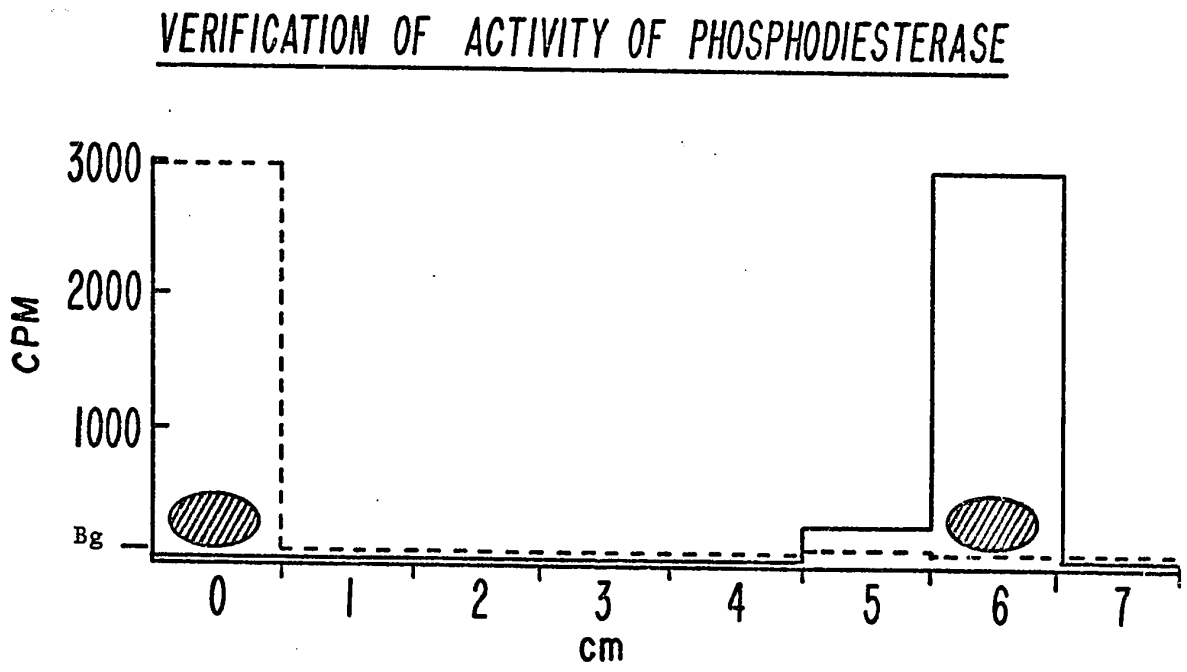


Fig. 3 Thin-layer chromatography, performed under the same conditions as that in fig. 2. Two incubation mixtures were chromatographed. The first one (unbroken line) contained 100 µµmol of cyclic AMP, 15 000 CPM [^{3}H]-cyclic AMP and Tris-imidazole buffer, pH 7.8 in a total volume of 50 µl. The second incubation of the same volume and composition contained in addition 1 µl of phosphodiesterase. The incubation time was 20 minutes at 38°C and the incubation stopped by boiling. Aliquots of 10 µl of both incubation mixtures were chromatographed. Unlabelled cyclic AMP and 5'-AMP (1 µg/1 µl) were run in parallel. Spots of unlabelled nucleotides were visualized under UV and the radioactivity was detected by counting 1 cm strips in a liquid scintillation spectrometer. Bg=background.

the urine was passed through a Millipore^{R} filter 0.45 µm, sterile form, to separate solid particles, cells and bacteria. Then, 0.24 ml of 0.3 N $Ba(OH)_2$ and 0.16 ml of 0.3 N $ZnSO_4$ (quantities that were found to neutralize each other) were added to the filtrate. After centrifugation at 10 000 x g for 5 minutes, the supernatant was applied on a 3 cm high column of Dowex 50 mounted in a Pasteur pipette. The column was eluated with water and the fraction contained between 1 and 4 ml of the eluate conserved and lyophilized. The lyophilized powder was dissolved in 0.4 ml of Tris-$MgCl_2$ buffer and 50 µl were separated for the recovery counting.

Krishna reported that the inorganic phosphate can be eliminated by Zn-Ba precipitation (134). We verified the efficacity of this procedure by a semi-quantitative method, based on the precipitation of inorganic phosphate by triethylammine and ammonium molybdate (256). This reaction produced a white cloudy precipitate containing only 3 mg% of PO_4^{---}. A yellow better formed precipitate is produced with higher concentration of phosphate. Our results, as shown in table 5, confirmed that the interference from the physiologically present concentration of inorganic phosphates in human urine will be quantitatively eliminated with only one Zn-Ba precipitation.

d - *Recovery of urinary cyclic AMP*

As previously mentioned, [^{3}H]-cyclic AMP was added to the urine without any preliminary purification procedure. The recovery of the standard was measured by liquid scintillation counting of an aliquot of 0.25 ml of urine in 15 ml of Aquasol^{R} scintillation liquor (purchased from New England Nuclear) in a Tri-carb liquid scintillation spectrometer, Packard model 3375. Another aliquot of 50 to 100 µl of resuspended lyophilized powder obtained at the end

TABLE 5

Elimination of inorganic phosphate by $Ba(OH)_2$-$ZnSO_4$ precipitation

PO_4^{--} dilution (g/liter)	physiological range 0.75	1.5	3.0	6.0	7.5	9.0	10.5	12.0	13.5	15.0
Before Ba-Zn precipitation*	clear	clear	cloudy	heavy yellow precipitation						
After Ba-Zn precipitation*	clear	clear	clear	clear	clear	cloudy	heavy yellow precipitation			

* To each different phosphate dilution in 5 ml, before and after Ba-Zn precipitation, 1.75 ml of the following mixture were added: 1 N $HClO_3$, 0.08 M NH_4Mb and triethylamine 0.2 M (4:2:1/vol) (256) and the resulting turbidity was estimated 10 minutes later

of the purification procedure was checked for the content of radioactivity in the same manner. An internal standard was provided by addition of 10 000 DPM of [^{3}H]-toluene to each sample for the correction for high quenching due to the yellowish color of urine (204). Results were calculated in DPM using the following equations (216):

(1) $$Efficiency = \frac{CPM\ internal\ standard + sample - CPM\ sample}{DPM\ internal\ standard\ *}$$

(2) $$DPM\ sample = \frac{CPM\ sample - background}{Efficiency}$$

e - *Enzymatic reactions*

The Aurbach and Houston method is based on a series of enzymatic reactions, transforming first cyclic AMP into ATP, then estimating the ATP formed by the radioactive phosphate exchange reaction. The first part, i.e. the enzymatic transformation of cyclic AMP into ATP, was originally described by Breckenridge (21) and the second part, by Glynn and Chappell (89). The enzymatic steps are as follows:

(1) 3', 5'-cyclic AMP $\xrightarrow[\text{phosphodiesterase}]{(Mg^{++})}$ 5'-AMP

(2) 5'-AMP + phospho-enol-pyruvate $\xrightarrow[\text{myokinase + pyruvate kinase}]{(ATP)}$ ATP

* DPM of internal standard were calculated by estimating the efficiency of the machine which was verified every four months by establishing a curve with the company tritium standards (Packard^R).

(3) $ATP + 3\text{-}phosphoglycerate + [^{32}P] \underset{phosphoglycerate\ kinase}{\overset{glyceraldehyde\ phosphate\ dehydrogenase}{\rightleftharpoons}} ATP^{32}$

After the completion of enzymatic reactions, the labelled ATP is in equilibrium with unlabelled ATP, radioactive phosphorus and unlabelled phosphates. The labelled phosphates must be eliminated by precipitation.

In this study, the precipitation by triethylammine hydrochloride and ammonium molybdate under acid conditions was used as recommend by Aurbach and Houston. This precipitation, separating inorganic phosphates from phosphoric acid esters, like ATP, was described by Sugino and Miyoshi in 1964 (256).

During the initial stage of the adaptation of this method, we were unable to reproduce the entire sequence of reactions. Initially, the conditions necessary for labelling of ATP (reaction 3) and for the separation of free radioactive phosphate were established and only then, the conditions for the second and finally the first reaction. This rather complicated approach was necessitated by the fact that it was quite impossible to find commercially available enzymes with the same activities as reported by the authors of the original method. The use of larger quantities of enzymes resulted in an increase in blank value due to the contamination of the enzymatic preparation by 5'-AMP (114, 90). An effort was made to set up the reactions in such a way that the maximum efficiency was obtained with the minimum quantity of the enzyme. Standard curves for each reaction with dilutions of ATP, 5'-AMP and finally cyclic AMP were established and are represented in fig. 4.

Several types of blanks were studied to establish the effects of omission of: 1) phosphodiesterase, 2) other enzymes, 3) cyclic AMP, 4) radioactive phosphate, 5) incubation mixture with the exception of $[^{32}P]$. It was found that the

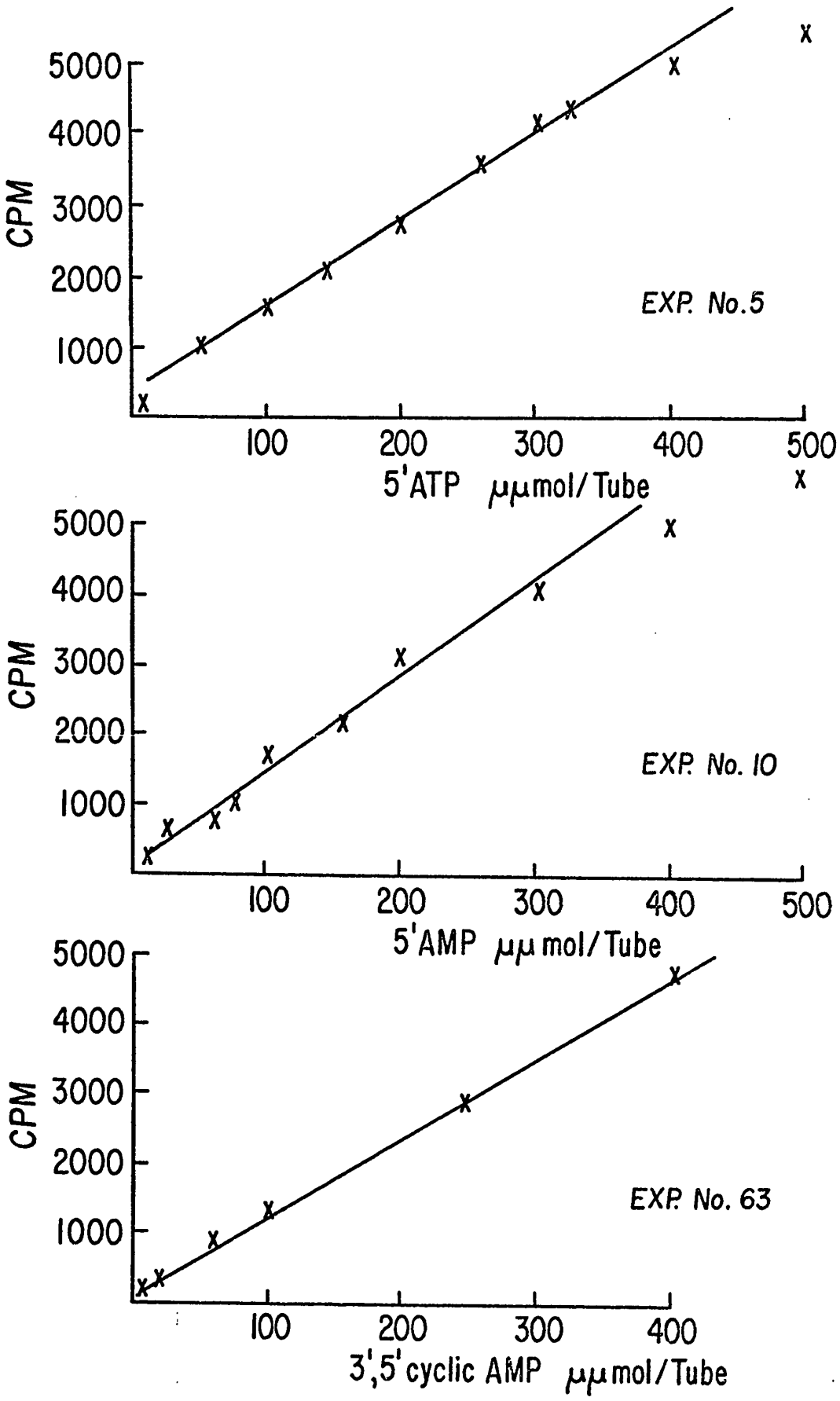


Fig. 4: Standard curves for ATP, 5'AMP and cyclic AMP as established in course of development of enzymatic assay.

omission of phosphodiesterase permits the evaluation of the specificity of the reaction; the omission of other enzymes and that of cyclic AMP establishes the importance of the unspecific binding of [^{32}P] on proteins and the importance of the contamination of enzymes by 5'-AMP; the omission of [^{32}P] is useful for the reading of background radioactivity; and the omission of the incubation mixture with the exception of [^{32}P] establishes the efficiency of the precipitation of phosphates (radioactive and unlabelled). The relative importance of these blanks is shown in table 6. The blank with the omission of phosphodiesterase was considered to be the most important and was therefore used for routine work (refer to calculations).

The enzymatic reactions were realized in two consecutive incubations. The incubation mixtures for routine work were of the following composition: *first incubation mixture* contained 1 µl of phosphodiesterase in 50 µl of 50 mM Tris-HCl at pH 8.0, containing 1.8 mM $MgCl_2$ and 0.1 mg/ml of albumine. The incubation started with the addition of 25 to 50 µl of a standard or an unknown dilution of cyclic AMP. *The second incubation mixture* consisted of a total volume of 50 µl containing Tris-HCl, pH 8.0, 160 mM; albumine, 0.1 mg per ml; EDTA, 0.2 mM; pyruvate kinase*, 8 µg per ml; phosphoglycerate kinase*, 20 µg per ml; myokinase*, 60 µg per ml; glyceraldehyde phosphate dehydrogenase*, 200 µg per ml; phospho-enol-pyruvate, 0.18 mM; 3-phosphoglycerate, 0.4 mM; cystein-free base, 4 mM; magnesium chloride, 9.6 mM; potassium chloride, 80 mM; ATP, 2×10^{-10} M; and [^{32}P], 2×10^{6} CPM per ml.

The first incubation lasted for 20 minutes at 37°C in a Dubnoff shaking incubator. Samples were then boiled for 3 minutes and kept on ice for 5 minutes. Then the second incubation mixture was added and after an incubation

* The quantity of enzymes must be determined for each batch.

TABLE 6

Relative importance of blanks in enzymatic measurement of cyclic AMP

PHOSPHODIESTERASE	OTHER ENZYMES	CYCLIC AMP	^{32}P	INCUBATION MIXTURE	PRECIPITATION OF Pi	CPM*
-	+	+	+	+	+	216
+	-	+	+	+	+	85
+	+	-	+	+	+	292
+	+	+	-	+	+	25
-	-	-	+	-	+	83
+	+	+	+	+	-	27 616

for 30 minutes at room temperature, the reaction was ended by cooling on ice. The precipitation of phosphate was carried out similarly as described by Aurbach with the following modification: the "carrier" solution of 0.32 mM inorganic phosphate (0.95 ml) was first added to the sample separately and well mixed. When this was followed by the addition of 0.35 ml of perchloric acid, 570 mM, ammonium molybdate, 23 mM, triethylamine hydrochloride, 29 mM, pH 5.0, and well mixed again, a heavy yellow precipitate appeared immediately. At this point, samples were left on ice for 10 minutes to complete the precipitation. After centrifugation in the cold for 10 minutes at 8000 x g, 0.2 ml of the resulting supernatant were added to 12 ml of scintillating solution (Permablend II^{R} (Packard) 6.6 g, toluene, 667 ml and Triton X^{R}, 333 ml) for the measurement of the unprecipitated portion of radioactivity. In our modification of the procedure, the acid-molybdate-triethylamine precipitating solution is able to eliminate 99.66±0.08% SE (mean of 14 experiments) of inorganic phosphate.

The standard concentrations of cyclic AMP (from 5 to 200 μμmol per tube) were used in duplicate in each experiment. Blanks (in duplicate) without phosphodiesterase and with 100 μμmol of cyclic AMP per tube were also used in every run. The purified samples of urine were resuspended in 0.4 ml of a mixture consisting of 40 mM Tris-HCl, pH 7.4, 40 mM imidazole and 1.8 mM magnesium sulfate. 50 μl of the above solution were run in duplicate with and without phosphodiesterase. Another control was routinely done by incubating a mixture of half of the volume of the unknown (25 μl) with half volume of the standard (25 μl).

f.- *Calculation*

Samples were counted by liquid scintillation as described above for 10 minutes and the mean count per minute was used for calculations. The first step in calculations was the computation for the means of duplicates of standards and of unknowns with a subsequent subtraction of blanks and so obtaining the net count. The later value was then traced versus concentration using ordinary millimetric graph paper. Standard curves of 5 to 250 µµmol per tube of cyclic AMP, i.e. concentration of 5 to 250 x 10^8 M project a straight line. This characteristic permits computerization of the results using a linear regression method of least squares with the equation $y=kx+c$ on Programma 101^R (Olivetti). Our program gives as a result the intercept y and slope k of the regression equation. The unknowns may be directly computed after the standard curve is registrated in the machine memory. The procedure and the program are presented in tables 7 and 8. The evaluation of the error may not be calculated automatically and the procedure used is also outlined in table 7.

Results in µµmol per tube are corrected for a dilution factor and for a recovery factor. The excretion of cyclic AMP during the four hour period of our experiment is obtained from a multiplication by the volume and, when indicated, corrected for excretion per g of creatinine.

2 - RADIOIMMUNOASSAY

a - *Material*

In order to simplify the procedure, the radioimmunoassay described by Steiner et al. in 1969 (252) was adopted for the measurement of cyclic AMP.

TABLE 7

Programma 101 procedure for computing the results of enzymatic measurement of cyclic AMP.

1.0 Compute the mean CPM of standard duplicates, unknown sample duplicates and blank duplicates from Tricarb scintillation sheet.
1.1 Subtract the value of standard blank from CPM of standards and a particular blank from each unknown.

2.0 Load Program I (table 8) on the machine. Set the decimal wheel at "4".
2.1 Press "V".
2.2 Enter y of the first standard (in CPM). Press "S".
2.3 Enter x of the first standard (in μμmol). Press "S".
2.4 Repeat step 2.2 and 2.3 for all y and x of standard dilutions.
2.5 Press "Z".
2.6 Machine prints out y intercept as A ◊ and k slope as B ◊. Enter them on Tricarb scintillation sheet.

3.0 Press "Y".
3.1 Enter again the y (in CPM) of all standards. Machine prints out the concentration x as found on computerized standard curve. Compare the reliability of various regions of curve between computerized and measured results. Proceed to the next step when differences are "acceptable". (An arbitrary limit of a 10% difference was adopted as "acceptable").

4.0 Enter y (in CPM) of unknowns. Results are in μμmol per tube.

5.0 Calculate concentration per ml, correct for recovery and calculate the excretion per volume excreted and/or per gram of creatinine.

TABLE 8

PROGRAM 1

```
V
b *
B *
c *
C *
d *
D *
B V
/ ◊
b ↓
  S
  +
b ↕
  ↓
  S
  X
c ↕
  +
c ↕
  ↕
B ↕
  +
B ↕
A X
C +
C ↕
d ↓
e +
d ↕
C V
A Z
```

```
c ↓
A X
D ↕
C ↓
d X
D -
D ↕
B ↓
c X
C ↕
b X
C -
D ÷
/ ◊
A ◊
b ↕
c X
B ↕
d X
B -
D ÷
B ↕
B ◊
C V
A Z
A Y
  S
  ↓
b -
B ÷
A ◊
  Y
```

The 2'O-succinyl derivative of cyclic AMP, following its binding to human serum albumine, was prepared in our laboratory and used as an antigen to develop the antibody in the rabbit. Since the study on the properties of this antibody was not terminated at the time of writing of this thesis, a detailed description of the procedure which was identical to that described by Steiner et al., will be omitted. The antibody ultimately used for the determination of cyclic AMP presented in this study was purchased from Collaborative Research, Waltham. This material was dispatched on dry ice in a 1/200 to 1/3000 titer. The competitor, the radioactive [^{125}I]-succinyl cyclic AMP-tyrosine methyl ester, was also purchased from Collaborative Research. The goat anti-rabbit IgG was purchased from Miles, Kankakee, Ill.

b - *Antigen-antibody reaction*

The reaction between the antibody and the labelled antigen is as follows:

(1) $[Ab] + [Ag^*] \rightleftharpoons [AbAg^*]$

The reaction represents the binding of the antibody, with the radioactive antigen, in this case [^{125}I]-succinyl cyclic AMP-tyrosine methyl ester; the antibody is a gamma globulin obtained from rabbits after immunization with succinyl derivative of cyclic AMP. The complex [AbAg*] is in equilibrium with [Ab] and [Ag*]. This basic relationship has been established in 1907 when Arrhenius applied the *Law of Mass Action* to the reaction between the combining sites of an antibody and its specific antigen (112). The term "avidity" is used to denote the energy of this reaction and is essentially the same as the association constant [K] in physical chemistry:

(2) $$K = \frac{[AbH]}{[Ab]\ [H]}$$

where [AbH], [Ab] and [H] refer to the molar concentration of the antibody hormone complex, free antibody and free hormone respectively. When we add to the reaction medium (1) the unlabelled hormone, in our case cyclic AMP (as a standard or unknown), the competition for antibody binding sites establishes a new equilibrium:

(3) $[Ab] - [^{125}I\text{-}c\text{-}AMP] - [c\text{-}AMP] \rightleftharpoons [Ab\ ^{125}I\text{-}c\text{-}AMP] - [Abc\text{-}AMP]$

The standard immunoassay procedure was as follows: anti-succinyl cyclic AMP rabbit antibody (globulin fraction) was diluted sufficiently to retain at least 30% of the radioactivity of the iodinated derivative of cyclic AMP after precipitation in presence of an excess of goat anti-rabbit IgG. 100 µl of the diluted anti-succinyl cyclic AMP antibody was added to 300 µl of either a diluted urine sample or 0.05 M sodium acetate buffer, pH 6.2, containing a nonradioactive standard dilution of cyclic AMP. A 100 µl aliquot of a solution of [^{125}I]-succinyl cyclic AMP tyrosine methyl ester diluted to contain 4000 to 8000 CPM (0.05 to 0.1 µµmol) were then added to bring the total reaction volume to 0.5 ml. The tubes were incubated for three hours at 4°C for complete equilibration of the competitive reaction.

c - *Separation of "free and bound" fraction*

Several modes for separation of "free and bound" antigen are described in the literature (111). A very simple procedure, described by Weinryb et al., consisting in a separation without precipitation of antibody using Millipore filter was first tried in this study (281). The separation of bound and unbound fractions by cellulose ester filters, reported by these authors, requires, unfortunately, a high concentration of the precious antibody for the

adsorption on the filter. More satisfactory results were obtained when the bound antigen was separated by ammonium sulfate precipitation as described by Chard et al. (36). In this method, a half saturated solution of ammonium sulfate precipitates the bound fraction in the presence of horse gamma globulin as a "carrier protein". The procedure was fairly satisfactory but the instability of the bound complex and a poor reproducibility were the drawbacks. Therefore, the original mode of separation by second antibody precipitation described by Steiner et al. was retained.

After the first incubation (described in the preceeding section), an excess of a second antibody (goat anti-rabbit IgG serum) was added and it was followed by an incubation overnight at $4^{o}C$. The optimal quantity of the second antibody (titer) must be determined for each lot of purchased preparation. A satisfactory precipitation was usually obtained with 5 to 25 µl of an undiluted anti-IgG. After the second incubation period, samples were centrifuged at 7000 x g in cold for 20 minutes and the precipitate was washed once with 0.5 ml of cold 0.05 M sodium acetate buffer, at pH 6.2 and then the radioactivity was counted in a gamma spectrometer (Packard, model 3003).

Urine samples were analyzed without any preliminary purification. In fact, no difference was observed between the samples purified as described above for the enzymatic assay and those where the purification was completely omitted. Urine samples were however diluted 5 to 10 times with 0.05 M sodium acetate buffer, at pH 6.2, in an effort to allow the use of the straight segment of the standard curve. All analyses were performed in quadruplicate, each sample representing a different dilution, usually 1/100, 1/200, 1/250, and 1/500 of 1 ml of the original urine sample.

d - *Calculation*

The graphical representation of our standard curves is similar to those described by Weaver and Cargille (280). These authors showed that: 1) standard curves plotted as P (percentage of bound) versus X (concentration) on a linear scale are hyperbolic; 2) when P is plotted against X on a logarithmic scale (semilogarithmic paper), the shape of the curve becomes sigmoid; 3) and finally, a straight line is obtained when the percentage is transformed into *logit* scale. An example from our experiment is shown in fig. 5a). Ekins recommended an alternate mode of representing the data resulting in a straight line curve (62). In this modification, the radioactivity R is plotted on the ordinate in *log* scale after the computation of (R free/bound - R free/bound 0) against the concentration plotted along the abscissa which is in *log* scale as well. In our adaptation the formula is:

$$\frac{St - b_n}{b_n} - \frac{St - b_o}{b_o}$$

where St - total CPM of standard stock dilution of [^{125}I]-cyclic AMP derivative; b_n - net CPM of bound fraction of standard dilution or unknown; b_o - net CPM of bound fraction or sample with no competitor added; net CPM meaning after blank subtraction. The equation is computed with the help of Programma 101 and the results are plotted on a graph in a *full-log* scale with 3 x 3 cycles resulting in a standard curve having a progressive character. An example of the standard curve is shown in fig. 6 and the routine procedure for the calculation is presented in table 9 and programs in tables 10 and 11. The four dilutions of each sample are read on the standard curve renewed daily and then readjusted for 1 ml. The result is the mean obtained from the four above dilutions.

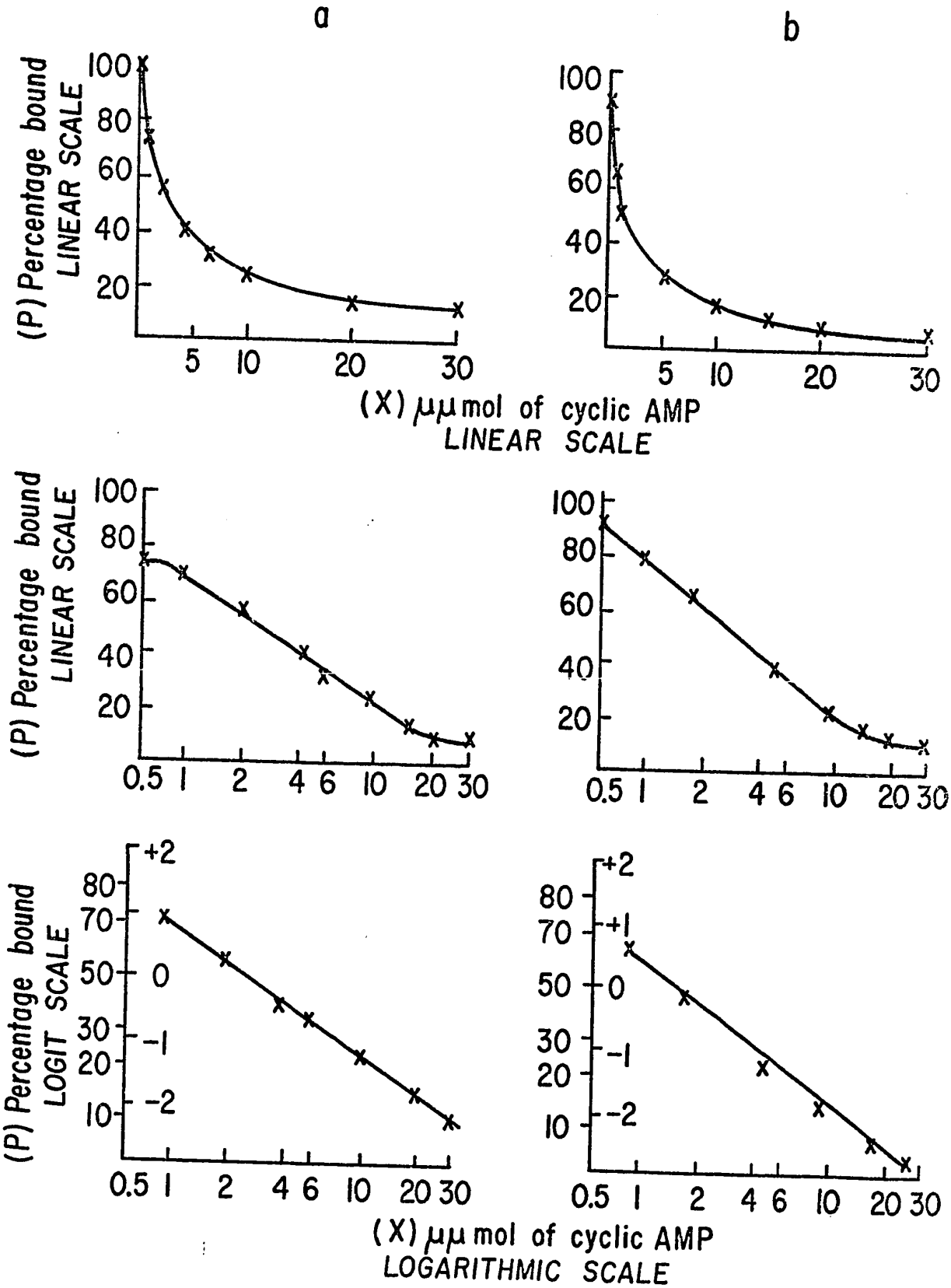


Fig. 5 Graphical presentations of the same standard curve for the radioimmunoassay (a) and for the protein binding assay (b).

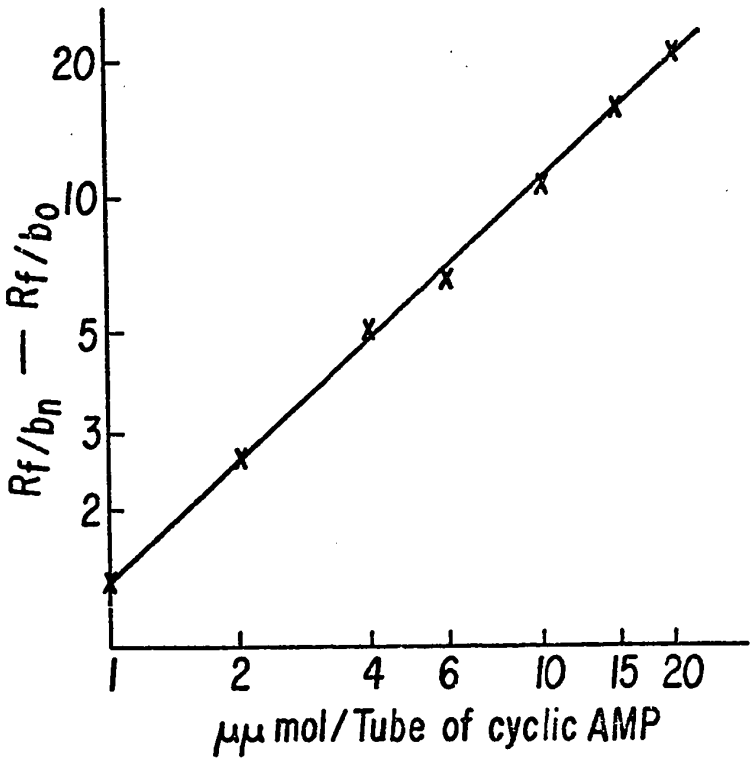


Fig. 6 -Standard curve for radioimmunoassay as computed from the formula

$$\frac{St - b_n}{b_n} - \frac{St - b_o}{b_o}$$

TABLE 9

Procedure for computerizing the results of radioimmunoassay.

1.0 Compute the mean of standard dilutions in CPM.

2.0 Press general reset.
2.1 Load Program II (table 10) on Programma 101. Set the decimal wheel at "4".
2.2 Press "V".
2.3 Enter blanks (dilution without binding protein) of standard curve in CPM. Press "S".
2.4 Enter the mean of standard dilution in CPM. Press "S".
2.5 Enter "O" (result of incubation without unlabelled competitor) in CPM. Press "S".

3.0 Press "W".
3.1 Enter CPM of bound fraction for the lowest standard dilution. Press "S".
3.2 Repeat step 3.1 for all standard dilutions.

4.0 Press "Y".
4.1 Enter blank (dilution without binding protein) of unknown sample. Press "S".
4.2 Enter CPM of first dilution of unknown. Press "S".
4.3 Repeat step 4.2 for other dilution of same unknown.

5.0 Repeat steps 4.0 to 4.3 for all unknowns.

6.0 From results of steps 3.1 and 3.2 draw a standard curve on *full-log* scale paper.
6.1 Read on this curve results for unknown values from steps 4.2, 4.3 and 5.0.

7.0 Load Program III (table 10) on Programma 101.
7.1 Press "V".
7.2 Enter values of unknowns as read on standard curve for each dilution. Obtain results of each dilution per ml.

8.0 Load Program IV (table 11) on Programma 101.
8.1 Press "V".
8.2 Enter values per ml. Press "W". Obtain mean and coefficient of variation between dilutions.

TABLE 10

PROGRAM II

olivetti underwood programma olivetti underwood programma olivetti underwood programma

PROGRAM III

olivetti underwood programma olivetti underwood programma olivetti underwood programma

TABLE 11

PROGRAM IV

underwood programma

olivetti underwood programma

olivetti

derwood programma

olivetti underwood programma

olivetti

3 - PROTEIN BINDING ASSAY

This assay was recently adopted in our laboratory in order to further simplify the measurement of cyclic AMP, increase the sensitivity and the accuracy of measurement in the plasma and lower the cost of materials. The protein binding assay was adopted in the form described by Gilman (88).

a - *Material*

Cyclic AMP-dependent protein kinase was prepared by the simplified procedure recommended by Gilman. This method involves acid precipitation followed by ammonium precipitation and separation on DEAE-cellulose column as originally recommended by Miyamoto et al. and Walsh et al. (186, 275).

Fresh rabbit muscle was obtained, immediately after bleeding of the animal, by cutting dorsal and leg muscles. About 250 g of muscle was homogenized in a Waring Commercial Blender with the pH adjusted to 5.5 and the precipitate removed by centrifugation. The supernatant was adjusted to pH 6.8 with 1 M potassium phosphate buffer at pH 7.2 and then fractionated by the addition of 32.5 g of ammonium sulfate per 100 ml of protein solution. The precipitate, collected by centrifugation, was dissolved in 25 ml of 0.005 M potassium phosphate buffer at pH 7.0, containing 0.002 M EDTA and dialyzed extensively against the same buffer. The protein solution was centrifuged at 78 000 x g for one hour and the precipitate discarded. This fraction was applied to a column of DEAE-cellulose (Whatman DE 11, 1 mEq/g; 24 x 4.5 cm) which was previously equilibrated with 5 mM potassium phosphate at pH 7.0 and then the column was washed with the same buffer. The first eluate was 600 ml of 100 mM potassium phosphate at pH 7.0 followed by 300 mM potassium

phosphate. The second eluate was collected in fifty 12 ml-fractions which were dialyzed against a 5 mM potassium phosphate buffer at pH 7.0 and then assayed for cyclic AMP binding activity. Fractions containing the highest activity (sixth to fourteenth) were pooled and used for the assay of cyclic AMP. The binding activity of various fractions is shown in fig. 7 and the effect of different quantities of this preparation on the binding of cyclic AMP on fig. 8.

The protein kinase inhibitor was prepared from rabbit muscle by homogenizing the tissue in 10 mM Tris-HCl at pH 7.5 and boiling it for 10 minutes. After the removal of the solid by filtration, the activity was precipitated with 1/9 volume of 50% trichloroacetic acid. The precipitate collected at 15 000 x g was dissolved in water and the pH adjusted to 7.0 with 1 N NaOH. This fraction was dialyzed against distilled water at room temperature, and the formed precipitate discarded. The inhibitor preparation was used at this stage of purity. The effect of different quantities of this preparation on binding activity of protein kinase is shown in fig. 8.

[^{3}H]-cyclic AMP at a high specific activity of 24.3 Ci/mmol was obtained from New England Nuclear. The radiochemical purity was verified as described in part III-B-1-a and shown in fig. 2. Glass fiber filters with a porosity of 0.45 µm and a diameter of 24 mm, were obtained from Millipore, Montreal.

b - *Principle and procedure of the assay*

The theoretical principle of the protein-binding assay is analogous to that of radioimmunoassay (refer part III-B-2-b). The important difference however is that the protein, binding cyclic AMP, is a natural, ubiquitous protein kinase. Binding of the nucleotide to the receptor part of this enzyme is enhanced in the presence of an acid-resistant protein first described

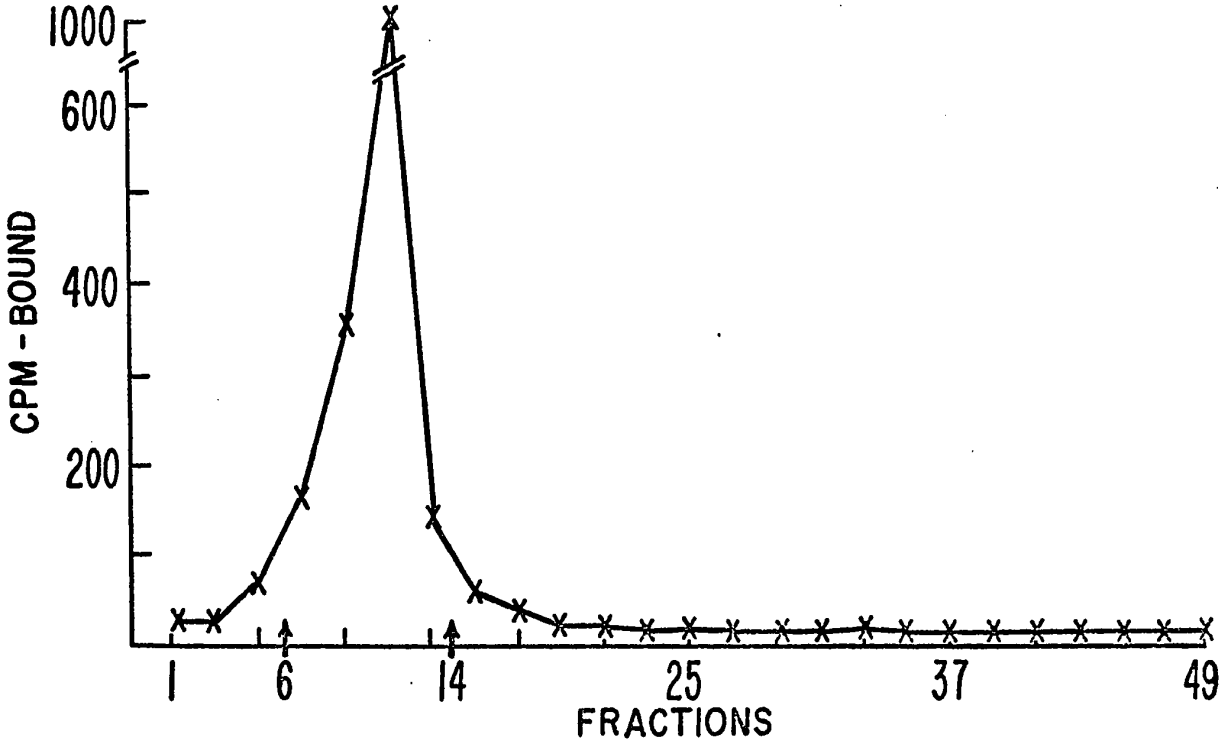


Fig. 7 Binding of [^{3}H]-cyclic AMP with dialyzed fraction of eluates from DEAE-cellulose chromatography. Fractions (of 12 ml each) 6 to 14 were collected together and used as protein kinase for measurement of cyclic AMP

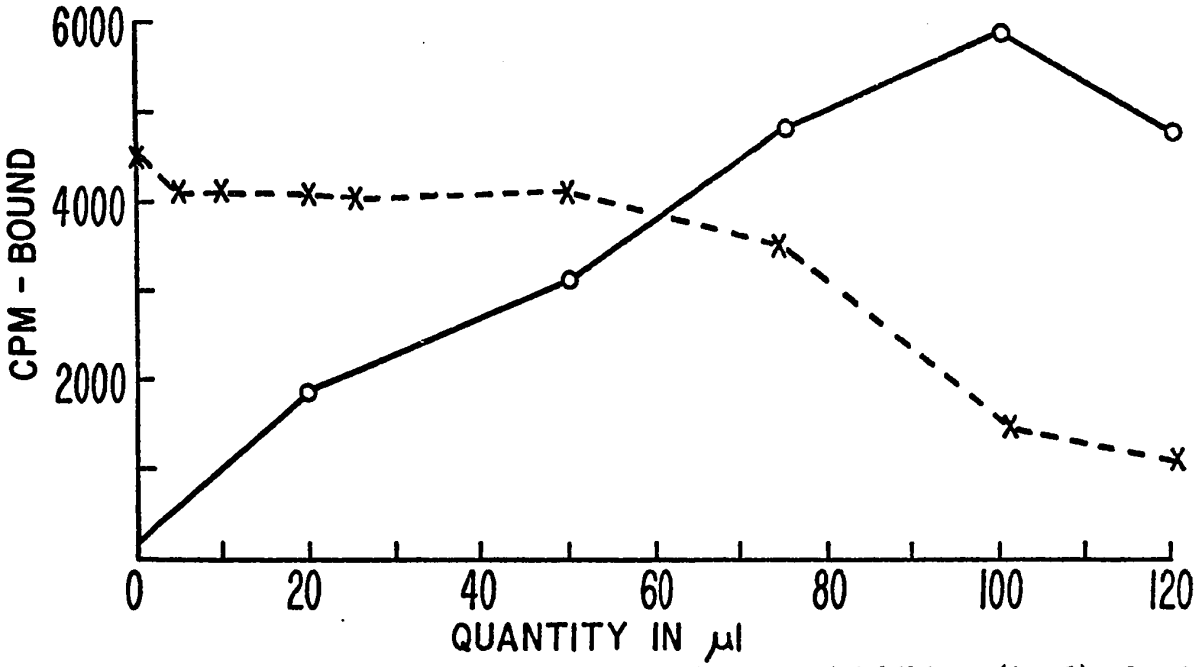


Fig. 8 Effect of different quantities of protein kinase and inhibitor (in µl) of original preparation on cyclic AMP binding. o———o represents the effect of different quantities of protein kinase; x---------x represents the effect of quantitative variation of the inhibitor. The total radioactivity of [^{3}H]-cyclic AMP was 18000 CPM in both experiments.

by Appleman (4). The effector part of the protein kinase is inhibited by this acid-resistant protein, but this inhibition of the enzyme activity is irrelevant for the assay. The second important difference in this assay, as compared to radioimmunoassay, is that the separation of "bound" and "free" material can easily be achieved by adsorption on a Millipore filter. The principle of adsorption on a Millipore filter was described before by Yarus and Berg in 1970 (288). Finally, assuring safety and low cost of this method, an adequate competitive reaction is achieved with tritiated nucleotide.

The standard binding reaction was conducted in a volume of 50 to 200 µl in 50 mM sodium acetate/acetic acid at pH 4.0. The other components of the incubation mixture were: [^{3}H]-cyclic AMP, 0.5 to 1.0 µµmol per 50 µl, with a protein titer sufficient to bind less than 30% of labelled nucleotide (usually 50 to 120 µl of our preparation), and where indicated, the maximum effective concentration of the protein kinase inhibitor preparation. Dilutions of standards and of the unknown samples of cyclic AMP were added following dilution in the same buffer. The reaction was initiated by the addition of the binding protein and blanks established by incubating the incubation mixture and the nucleotide with the omission of binding protein. After incubation for 60 minutes at 0°C, the samples were diluted to 1 ml with cold 20 mM potassium phosphate at pH 6.0 and 4 to 5 minutes later, they were placed onto a Millipore filter previously rinsed with the same buffer. After deposition of samples, the filters were washed with 10 ml of the same buffer and placed in counting vials containing 12 ml of scintillating solution (Aquasol^R, New England Nuclear). The content of radioactivity was estimated in a Tri-carb

scintillation spectrometer. The blanks, in the absence of binding protein, were 50 to 75 CPM. The presence of a filter in the scintillating solution does not alter significantly the efficiency of counting.

c - *Calculation*

Similarly to the radioimmunoassay, the graphical representation of the standard curve of protein binding corresponds to an hyperbola, a sinusoid or a straight line, depending on which scale or mathematical transformation are applied. An example is given in fig. 5b). The computing procedure was based on the equation suggested for saturating assays by Rodbard (223)

$$y' = \frac{100}{y} - 1; \quad y' = k_1 X,$$

and corresponding to the characteristic of a rectangular hyperbola. For the calculation of the slope and data interpolation by this equation, we used the program kindly supplied by Dr D. Rodbard (program VI, table 13). With this method of computation, which agrees very well with the graphical representation, we were able to program all calculating procedures (manual daily drawing of standard curve was unnecessary). The routine procedure for calculation is presented in table 12 and programs in tables 13, 10, 11.

4 - COMPARISON OF THE THREE METHODS

a - *Intra- and inter-assay variations*

The intra-assay variation was established by calculating the coefficient of variation between duplicates, in the case of the enzymatic assay, and between different dilutions (four) in case of radioimmunological and protein bind-

TABLE 12

Procedure for computerizing the results of protein binding assay.

1.0 Press general reset.
1.1 Load Program V (table 13) on Programma 101. Set the decimal wheel at "4".
1.2 Press "V".
1.3 Enter blank (dilution without binding protein) of standard curve in CPM. Press "S".
1.4 Enter standard dilution in CPM. Press "S".
1.5 Enter "0" (result of incubation without unlabelled competitor) in CPM. Press "S".

2.0 Press "W".
2.1 Enter CPM of bound fraction for the lowest standard dilution. Press "S". Obtain the percentage of bound fraction (Y).
2.2 Repeat step 2.1 for all standard dilutions.

3.0 Press "Y".
3.1 Enter blank (dilution without binding protein) of unknown sample. Press "S".
3.2 Enter CPM of first dilution of unknown. Press "S". Obtain the percentage of bound fraction (Y).
3.3 Repeat step 3.2 for other dilutions of the same unknown.

4.0 Repeat steps 3.0 to 3.3 for all unknowns.

5.0 Load Program VI (table 13) on Programma 101.
5.1 Press "V" (clears). Press "Y" (read for use).
5.2 Enter Y_1 (percentage of bound fraction) of standard curve.
5.3 Enter X_1 (dilution of unlabelled cyclic AMP in µµmol/tube).
5.4 Repeat 5.2 and 5.3 for all Y and X of standard curve.

6.0 Press "W". Get slope k.
6.1 Enter 1 F ↑ (or other multiplier constant).

7.0 Press "Z". (ready for interpolation).
7.1 Enter Y of standard, get X from machine standard curve. Check the reliability of various regions of curve between computerized and measured results (refer step 3.1 from table 7).
7.2 Enter Y of unknowns. Obtain X in µµmol/tube for each dilution.

8.0 Load Program III (table 10) on Programma 101.
8.1 Press "V".
8.2 Enter X of unknowns obtained in step 7.2. Obtain results of each dilution per ml.

9.0 Load Program IV (table 11) on Programma 101.
9.1 Press "V".
9.2 Enter values per ml. Press "W". Obtain mean and coefficient of variation between dilutions.

TABLE 13

PROGRAM V

olivetti underwood programma

olivetti underwood programma

olivetti underwood programma

```
V
S
↓
B↕
S
↓
B -
F ÷
C↕
AW
S
↓
B -
C ÷
A◊
W
AY
S
↓
B↕
AZ
S
↓
B -
C ÷
A◊
Z
S
100•0000 F◊
```

PROGRAM VI

olivetti underwood programma

olivetti underwood programma

olivetti underwood programma

```
V
B*
C*
AY
e↓
S
÷
e X
e -
e ÷
B•
B↕
S
↓
C•
C↕
/◊
Y
AW
B↓
C ÷
A◊
D↕
AZ
e↓
S
÷
e X
e -
e ÷
D ÷
F X
A◊
/◊
/◊
Z
S
100•0000 e◊
```

ing assays. It is important to note that the mean of duplicates or different dilutions are presented as a result, actually decreasing the real error due to sample to sample variation. The respective intra-assay variations of the three methods are presented in table 14.

The inter-assay variations are established by calculation of the coefficient of variation between the results obtained by different methods for the same sample. From this evaluation, presented in table 15, it is evident that the lowest variation and the highest correlation exist between radioimmunological and protein binding assay. The absolute values measured by these three methods are however different, mainly because of a high coefficient of variation of the enzymatic method. Reasons for variations with this method are discussed below (presence of an enzymatic inhibitor in urine). Since the correlations between the three methods are significant, the results obtained by each individual method can be considered as reliable.

b - *Interference, simplicity, cost*

Many possible interferences must be taken into account in the measurement of urinary cyclic AMP with the enzymatic assay. The first is the presence of phosphates in urine, which can substantially lower the specific activity of [^{32}P] in the exchange reaction. Phosphates were therefore eliminated by Zn-Ba precipitation and Dowex chromatography. Secondly, when the enzymatic method was used, it was observed that higher results were obtained, for the same sample, with a higher dilution. The inhibitory effect of concentrated urine was not abolished either by Zn-Ba precipitation or Dowex column chromatography. The inhibitory factor was absent when the standard dilution of cyclic AMP was purified, involving that this effect was not due to different

TABLE 14

Intraassay coefficient of variation of assays used for cyclic AMP determination

ASSAY	CALCULATED FROM	NUMBER OF MEASUREMENT	COEFFICIENT OF VARIATION (in %)
ENZYMATIC	DUPLICATES	78	22.35
RADIOIMMUNOASSAY	FOUR DILUTIONS	85	8.49
PROTEIN BINDING	FOUR DILUTIONS	30	6.84

TABLE 15

Comparisons between assays used for cyclic AMP measurement

ASSAYS COMPARED	ENZYMATIC RADIOIMMUNOASSAY	ENZYMATIC PROTEIN BINDING	RADIOIMMUNOASSAY PROTEIN BINDING
Number [1]	17	15	30
Coefficient of variation	43.12%	40.55%	14.62%
Coefficient of correlation	0.71	0.59	0.98
t of coefficient of correlation	3.86*	2.53*	25.09***

[1] Number of samples measured by the two methods compared

* Significant at the level of 95%

***Significant at the level of 99.9%

stages of purification. Boiling and filtration through 0.45 µm Millipore established that the inhibitory factor is heat stable and not particulate. It is probable that the observed inhibitor of the enzyme activity is similar to those described by Wilkinson and by Mattenheimer (285, 174). The dilution of urine samples up to 32-times was necessary to diminish the effect of this enzymatic inhibitor. Unfortunately, this unpublished fact was realized only late in our study and for a technical reason, the analysis of a part of samples could not have been repeated.

No such difficulties were encountered during the use of two assays with competitive binding principle. Since micro-organisms are able to produce their own cyclic AMP (197), urine samples for cyclic AMP measurement by any of the methods must be protected against bacterial contamination and growth. This was achieved by collecting urine samples on ice and keeping them frozen until the determination.

The difficulties encountered in setting up of the three assays are probably best reflected by the time required which was several months, weeks and days for the elaboration of the enzymatic method, the radioimmunoassay and the protein binding assay respectively. The routine procedure of measurement was also simplified by competitive assays. The most important simplification is the omission of the purification of urine involving Zn-Ba precipitation and Dowex column chromatography in both competitive methods.

The cost involved in these three methods showed the same decreasing character: enzymatic method > radioimmunoassay > protein binding assay. The protein binding assay cost is limited to the purchase of Millipore filters and tritiated cyclic AMP.

C - MEASUREMENT OF URINARY CREATININE

The procedure used in this study was a modification of the method of Folin, as described by Cooper and Biggs (49). Reagents used comprise three standard solutions: a solution of creatinine made by dissolving 1 g of dry creatinine in 1 l of 0.1 N HCl; a 1.175% solution of picric acid; 10% v/v NaOH.

The routine procedure was as follows: duplicates representing samples of urine of 0.25 ml and 0.5 ml were first made up to 1 ml with distilled water and placed in 100 ml volumetric flasks. Then, 10 ml of 1.175% picric acid was added, followed by 1.5 ml of 10% NaOH. The contents of the flasks were thoroughly mixed and allowed to stand for 15 minutes before dilution to 100 ml. After the contents have been thoroughly mixed again, the absorbancies of the colored solution were measured with a Coleman spectrometer at 520 mμ. Reagent blanks were prepared in the same manner except that water replaced the test solution. A standard curve was established by measuring the optical density of 0.1 to 1 ml (0.1 to 1 mg) standard solution of creatinine. The curve obtained is a straight line. The effect of standard solution on creatinine measurement in urine is shown in table 16. All samples were measured in duplicate and the mean result is considered. The coefficient of variation between duplicates was 2.86 ± 0.36% (SE) for 79 samples measured.

Urinary sodium and potassium were measured by flame photometry as a routine hospital procedure.

TABLE 16

Effect of standard solution of creatinine on creatinine measurement in urine

SAMPLE	OPTIC DENSITY	CONCENTRATION PER VOLUME	CONCENTRATION PER ml
	mμ	mg	mg
Urine I*	.122	0.15	0.60
Urine II	.24	0.31	0.62
Urine I + standard**	.15	0.20	0.80
Urine II + standard	.3	0.40	0.80

* Urine I and II represent 0.25 ml and 0.5 ml respectively

** 5 ml of urine were mixed with 5 ml of the standard dilution of creatinine (conc. 1 mg per ml); then 0.25 ml and 0.5 ml of mixture are processed.

D - STATISTICAL ANALYSIS

Analysis of variance by randomized blocks was used for the study of differences due to body position or isoproterenol infusion in the excretion of creatinine, electrolytes, pulse rate and blood pressure; each patients being considered as a block.

In the first part of our study, where a group of control subjects and two groups of patients were included, the Dunnett's test was used for multiple comparisons with a control (57). Different correlations were calculated by the Pearson's least-squares method. Paired or unpaired Student t test performed on the ratio of two dependent values may lead to bias results (166). Since the cyclic AMP excretion has a linear correlation with the creatinine excretion (refer results), our results were analyzed by a covariance analysis using the method of reduced Y (166).

An important problem was first to determine whether the difference in cyclic AMP excretion in recumbent and upright positions was itself significantly different between the two groups of subjects. Since this difference was positive in some subjects and negative in others, an arbitrary value had to be added to each of the differences to give them all the same sign. The so-modified data were subsequently submitted to Dunnett's test or Student t test as indicated. The same device was used with the pulse rate and the mean blood pressure. The same approach was used to compare the excretion of the nucleotide, pulse rate and mean blood pressure following isoproterenol infusion in reclining subjects with the same recumbent controls (refer tables 19, 25, 28, 50).

The data of cyclic AMP excretion were in addition analyzed by nonparametric statistical test, which is appropriate for the analysis of small samples or to

treat observations obtained from two possibly different populations regardless of the shape of distribution of the population (246). The tests used were those of Wilcoxon, one for two samples and the other for paired differences (246, 53). These methods utilize the information from the relative magnitude as well as from the direction of differences.

IV - RESULTS

A - CYCLIC AMP MEASUREMENT BY ENZYMATIC METHOD

1 - EFFECT OF POSTURE

Individual data on pulse rate and cyclic AMP excretion are shown in tables 17 and 18. The analysis of mean pulse rate by randomized blocks showed a highly significant increase in the upright position for each group.

Individual values of cyclic AMP excretion showed wide variations, from 0.1 to 6.7 µmol/g of creatinine. The response to upright posture was different in the three groups studied. In all control subjects, the excretion of cyclic AMP was lower during upright position (for differences in mean values, $p < 0.01$), but in most patients with labile hyperkinetic hypertension it was higher in the same position ($p < 0.01$). In patients with stable benign hypertension, the response was mixed; the mean response was near zero.

Table 19 demonstrates differences in two groups of patients, as compared to control subjects, studied by comparison of the amplitude of change in pulse rate and cyclic AMP excretion in upright position.

2 - DISCUSSION

This study provides us with a screening method for the detection of possible abnormalities in the "second messenger system" in human hypertension. The differences observed may seem unrealistically significant if large variations between individual results are considered. Many results seem also unusually low, in spite of the fact that the mean excretion with confidence limits in control subjects (refer table 17) correlates with the values reported by other workers (refer table 3).

TABLE 17

Clinical data and urinary excretion of cyclic AMP as measured by the enzymatic assay.

CONTROL SUBJECTS

		RECUMBENT			UPRIGHT		
AGE	SEX	PR†	MBP††	cAMP§	PR†	MBP††	cAMP§
21	M	84	106	0.9	116	95	0.7
21	M	87	76	2.2	92	83	1.8
21	M	91	97	1.5	101	85	0.2
22	F	82	69	4.9	74	76	3.4
22	M	78	93	2.3	74	84	1.3
22	M	64	83	3.9	71	85	1.5
22	M	52	73	1.3	57	78	1.0
23	M	60	87	1.1	84	99	0.1
24	F	69	82	6.7	78	85	5.0
24	F	74	72	4.8	85	78	3.9
25	M	66	104	4.2	86	89	2.1
27	M	68	82	1.2	72	88	0.3
27	M	55	72	0.8	65	80	0.3
Mean		72		2.7	81		1.7
CL_{95}		7.4		1.0	9.3		0.8

† Mean pulse rate per minute during 4 hours of study.

†† Mean of blood pressure in mmHg (diastolic + 1/3 of pulse pressure during the same period).

§ Urinary excretion of cAMP, expressed in micromoles per gram of creatinine, collected during the same period.

TABLE 18

Clinical data and urinary excretion of cyclic AMP as measured by the enzymatic assay.

LABILE HYPERKINETIC HYPERTENSION

AGE	SEX	SYMPTOMS*	RECUMBENT			UPRIGHT		
			PR†	MBP††	cAMP§	PR†	MBP††	cAMP§
18	M	1,3,5	81	87	1.3	108	93	2.6
20	M	1,3,5	71	91	1.5	85	93	1.4
22	M	1,3,4,5	71	79	0.7	99	102	2.2
22	M	1,5	58	92	0.5	85	83	1.3
23	F	1,2,5	87	104	1.3	96	94	1.7
23	M	5	78	95	1	111	88	1.9
25	M	1,4,5	89	103	1.7	108	89	2.8
27	M	1,5	76	87	0.6	104	104	0.9
27	M	1,3,4,5	68	108	0.4	96	121	1.2
28	F	1,2	85	129	2.3	110	126	2.0
37	F	1,2,4,5	97	121	0.7	97	135	1.2
45	F	1,5	74	101	2.8	73	91	2.6
50	F	4	102	94	1.5	134	92	2.7
Mean			80.		1.2	101		1.9
CL_{95}			7.4		0.4	9.1		0.3

STABLE HYPERTENSION

26	M	1,5	64	113	2.6	70	112	2.4
31	M	2,5	83	112	1.5	95	110	3.7
33	F		80	149	3.1	80	150	4.6
39	M	5,6	70	94	3.0	81	96	2.2
41	M	4	92	116	2.3	108	132	1.9
42	F	1	72	112	1.5	72	99	2.6
45	F	4,5	91	118	1.4	108	121	2.5
46	F	5	78	113	3.3	99	113	4.0
47	F	6	66	132	6.0	81	115	4.3
50	M	1,5	80	120	0.7	90	130	1.5
52	M		75	118	3.1	79	125	2.6
54	M	2,5	90	109	1.8	88	111	0.8
55	M	6	58	110	1.3	81	117	0.7
Mean			74		2.4	84		2.6
CL_{95}			5.7		0.7	5.7		0.6

* 1, dermographism; 2, dyspepsia; 3, proven gastroduodenal ulcers; 4, neurovegatative pattern of glucose tolerance test; 5, anxiety and/or depression; 6, suppressed plasma renin activity. (For explanations refer table 17)

TABLE 19

Changes of pulse rate and cAMP excretion with change to upright posture, modified to eliminate negative values.

PULSE RATE	MEAN	CL_{95}	*p*
Healthy subjects	90*	5.3	-
Hypertension: labile	79	5.7	0.05
stable	90	4.0	*N.S.*
cAMP EXCRETION			
Healthy subjects	11†	0.34	-
Hypertension: labile	9.3	0.23	0.01
stable	9.8	1.3	0.01

* Difference (pulse rate recumbent minus pulse rate upright) beats/minutes, plus 100.

† Difference (recumbent cAMP excretion minus upright cAMP excretion). micromoles per gram creatinine, plus 10.

Since the enzymatic method used showed an intraassay coefficient of variation of 22.35% (refer part III-B-4-a), the above reported results must be regarded with caution. Another bias is introduced by the fact that the presence of enzymatic inhibitors in urine was not taken into consideration at the beginning of this study. Finally, samples were not randomized before measurements of cyclic AMP to avoid the influence of day to day variation due to the variability of the procedure itself. It may so happen that these technical defects could have an additive character and therefore increase the experimental error (166).

In spite of all these difficulties, the importance of the finding in the first stage of the study, showing the decrease of cyclic AMP excretion in control subjects and its increase in patients with labile hyperkinetic hypertension in response to upright position, encouraged to continue this work. These preliminary results were further supported by findings obtained with a more precise procedure, the radioimmunoassay, for cyclic AMP determination. They are presented in the next chapter.

B - CYCLIC AMP MEASUREMENT BY RADIOIMMUNOASSAY

1 - STUDY OF POSTURE

a - *Effect on pulse rate, blood pressure, excretion of cyclic AMP and electrolytes.*

Individual data obtained from control subjects and patients with labile hyperkinetic hypertension are presented in tables 20 and 21. These data showed that the upright position increased the pulse rate in both groups of subjects, but statistical analysis demonstrated highly significant difference in pa-

TABLE 20

Basic data and cyclic AMP excretion-
effect of posture

CONTROL SUBJECTS

NAME	AGE	SEX	RECUMBENT						UPRIGHT					
			PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§
E.S.	21	M	87	76	3.04	1.55	3.95	2.54	92	83	0.83	1.58	5.72	3.61
M.E.	21	M	82	77	1.50	0.68	1.96	2.86	58	90	1.02	0.91	2.58	2.84
N.Y.	21	M	91	97	0.98	1.09	2.57	2.37	100	85	0.17	0.55	1.98	3.62
M.D.	21	M	57	72	6.42	1.17	1.94	1.67	65	80	1.90	1.00	1.48	1.48
P.S.	22	M	78	93	1.75	1.26	4.50	3.57	74	84	0.58	1.40	4.22	3.01
J.D.	22	F	82	69	3.60	0.76	3.92	5.17	74	76	1.23	0.64	3.69	5.72
R.D.	22	M	52	73	4.67	1.58	1.74	1.10	57	78	0.79	1.29	0.30	0.23
P.B.	23	M	60	87	2.35	1.20	2.83	2.36	84	99	0.25	0.83	0.54	0.65
N.T.	24	F	74	72	3.60	0.82	3.32	4.00	85	78	1.96	0.64	2.45	3.85
Y.D.	24	M	84	94	2.50	1.24	3.98	3.20	82	90	1.47	1.25	2.65	2.12
A.S.	25	M	66	104	4.12	2.37	11.94	5.02	86	89	1.15	1.66	6.53	3.93
M.G.	25	F	72	60	3.16	0.76	3.03	4.10	92	79	2.87	0.80	2.89	3.59
Mean			73.6	81.2	3.14	1.20	3.81	3.16	79.2	84.2	1.18	1.05	2.92	2.89
± SE			3.7	3.9	0.43	0.14	0.78	0.36	4.0	1.9	0.22	0.10	0.54	0.44

* Mean pulse rate per minute during four hours of study.
** Mean of blood pressure in mmHg (diastolic + 1/3 of pulse pressure) during the same period.
† Volume of urine in ml per minute during the same period.
†† Creatinine excretion in µg/min.
§ Cyclic AMP excretion in nanomol/min.
§§ Cyclic AMP excretion expressed in µmol and corrected per g of creatinine.

TABLE 21

Basic data and cyclic AMP excretion-

effect of posture.

LABILE HYPERKINETIC HYPERTENSION

NAME	AGE	SEX	RECUMBENT						UPRIGHT					
			PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§
J.G.	12	F	82	81	3.25	0.94	4.36	4.66	96	84	0.19	0.86	2.35	2.71
N.F.	15	M	81	96	3.56	1.41	3.89	2.90	93	73	0.57	1.24	4.46	3.60
A.C.	21	M	81	96	1.48	0.90	1.96	2.16	93	104	1.18	0.96	2.46	2.55
J.P.P.	21	M	84	106	2.16	1.27	1.76	1.38	116	95	1.26	1.00	3.16	3.13
P.D.	22	M	58	92	2.98	1.31	5.48	4.17	85	83	0.42	0.66	3.59	5.47
P.C.	27	M	68	108	6.70	1.41	2.67	1.88	96	121	3.06	1.22	2.30	1.88
J.L.	28	F	78	97	3.34	1.37	6.03	4.41	92	101	0.58	1.14	6.51	5.69
L.L.	29	F	68	121	4.48	0.94	4.94	5.25	84	111	3.54	2.05	9.82	4.78
G.P.	30	M	69	104	3.52	2.08	5.44	2.64	96	112	1.79	0.61	5.06	8.26
A.H.	30	M	67	100	3.29	1.14	1.32	1.16	76	101	0.41	0.60	2.72	4.53
G.L.	37	F	97	121	0.76	0.82	3.91	4.77	97	135	0.36	0.59	3.64	6.15
C.L.	37	F	77	93	3.04	0.95	2.49	2.61	78	88	0.86	0.69	2.17	3.12
J.C.A.	39	M	70	94	4.06	1.30	2.64	2.03	81	96	2.14	1.24	4.53	3.65
F.L.	40	M	84	109	1.83	1.83	7.13	3.89	91	103	1.11	1.74	6.97	4.01
Mean			76.0	101.3	3.17	1.26	3.86	3.13	91.0	100.5	1.24	1.04	4.27	4.26
± SE			2.6	2.9	0.38	0.09	0.47	0.36	2.7	4.3	0.27	0.11	0.59	0.45

For explanations refer table 20.

tients only (refer table 22). Creatinine excretion decreased slightly in both groups. The excretion of cyclic AMP in the upright position demonstrated: a small decrease (when corrected for the decrease of creatinine excretion in upright position) in control subjects, while a significant increase was observed in patients (refer table 23).

The following conclusion may be drawn from the analysis of the intergroup postural changes (table 24): 1) the pulse rate is slightly higher in recumbent patients, and a significant difference is oberved when the subjects assume the upright position; 2) the mean blood pressure is significantly higher in patients in either of the two positions; 3) the amount of cyclic AMP excreted in upright position is significantly higher in patients (with no difference between "baseline", i.e. recumbent values).

The results of the pulse rate, mean blood pressure and cyclic AMP excretion were analyzed with respect to the "amplitude of change" due to postural adaptation (data obtained by subtraction of upright values from the recumbent ones) (refer table 25). This analysis showed that the only significant difference induced by postural change is observed in the excretion of cyclic AMP.

Individual data of sodium and potassium excretion, as well as their ratio, summarized in tables 26 and 27, showed a highly significant decrease in the upright position in both groups of subjects (table 28). When the electrolytes excretion is compared between the two groups, it is evident that the sodium excretion is higher and the potassium excretion lower in patients when recumbent; differences are, however, statistically not significant(table 29) but the resulting sodium/potassium ratio shows a significant increase ($p < 0.02$).

TABLE 22

Statistical analysis of the effect of posture on basic data.

Analysis of variance by randomized blocks.

CONTROL SUBJECTS

	Source of variation			
	SUBJECTS		POSTURE	
	F	p	F	p
PULSE RATE	2.76	N.S.	2.12	N.S.
MEAN BLOOD PRESSURE	3.28	<0.05	1.10	N.S.
VOLUME	2.24	N.S.	26.06	<0.01
CREATININE EXCRETION	8.30	<0.01	3.89	N.S.

LABILE HYPERKINETIC HYPERTENSION

Source of variation			
SUBJECTS		POSTURE	
F	p	F	p
2.99	<0.05	31.4	<0.01
6.1	<0.01	0.07	N.S.
4.13	<0.01	42.43	<0.01
1.31	N.S.	2.25	N.S.

TABLE 23

Statistical analysis of the effect of posture on cyclic AMP excretion.

CONTROL SUBJECTS — LABILE HYPERKINETIC HYPERTENSION

Analysis of covariance (cyclic AMP per minute, reduced for creatinine excretion)

F	p
0.16	N.S.

F	p
4.32	<0.05

Paired Student t test (cyclic AMP per g of creatinine)

t	p
1.07	N.S.

t	p
-2.34	<0.05

Wilcoxon matched-pairs test (cyclic AMP per g of creatinine)

T	p
24	N.S.

T	p
17	<0.01

TABLE 24

Statistical analysis of the effect of posture on basic data and cAMP excretion between the two groups

Unpaired Student *t* test

	RECUMBENT		UPRIGHT	
	t	*p*	*t*	*p*
PULSE RATE	-0.54	N.S.	-2.51	<0.05
MEAN BLOOD PRESSURE	-4.17	<0.01	-3.24	<0.01
VOLUME	-0.05	N.S.	-0.17	N.S.
CREATININE	-0.33	N.S.	0.01	N.S.
CYCLIC AMP (PER g CREAT)	0.05	N.S.	-2.12	<0.05

Analysis of covariance (cyclic AMP per minute, reduced for creatinine excretion)

F	*p*	*F*	*p*
0.09	N.S.	-9.22	<0.01

Wilcoxon test for two samples (cyclic AMP per g of creatinine)

T	*p*	*T*	*p*
188	N.S.	128	N.S.

TABLE 25

Statistical analysis of changes of basic data and cyclic AMP excretion with change to upright posture (on data modified to eliminate negative values).

PULSE RATE*	*Mean*	S^2	$t^{\dagger}$	*p*
CONTROL versus	94.41	184.81	2.03	*N.S.*
PATIENTS	85.00	100.3		

MEAN BLOOD PRESSURE**	*Mean*	S^2	$t^{\dagger}$	*p*
CONTROL versus	96.91	113.5	-0.93	*N.S.*
PATIENTS	100.7	108.2		

CYCLIC AMP§	*Mean*	S^2	$t^{\dagger}$	*p*
CONTROL versus	10.27	0.79	2.45	<0.005
PATIENTS	8.88	3.18		
			$T^{\dagger\dagger}$	*p*
			214	<0.01

* Difference (pulse rate recumbent minus pulse rate upright) beats/min. plus 100.

** Same procedure as for pulse rate.

§ Difference (cAMP excretion recumbent minus cAMP excretion upright), micromoles/g of creatinine, plus 10.

† Unpaired Student t test

†† Wilcoxon test for two samples.

TABLE 26

Sodium and potassium excretion-
effect of posture

CONTROL SUBJECTS

NAME	RECUMBENT				UPRIGHT			
	VOLUME*	Na**	K†	Na/K††	VOLUME*	Na**	K†	Na/K††
E.S.	3.04	180	98	1.84	0.83	42	42	1.00
M.E.	1.50	103	36	2.86	1.02	98	35	2.80
Y.N.	0.98	72	67	1.07	0.16	4	17	0.24
M.D.	6.41	164	77	2.13	1.90	32	21	1.52
P.S.	1.75	108	67	1.61	0.58	21	54	0.39
J.D.	3.60	119	43	2.77	1.23	17	30	0.57
R.D.	4.67	173	87	1.99	0.79	42	26	1.62
P.B.	2.35	210	61	3.44	0.25	8	25	0.32
N.T.	3.60	105	68	1.54	1.96	-	-	-
Y.D.	2.50	85	80	1.19	1.47	62	72	0.86
A.S.	4.12	165	124	1.33	1.15	33	88	0.38
M.G.	3.17	92	60	1.53	2.87	75	52	1.44
Mean	3.14	132.2	72.3	1.94	1.18	39.4	42.0	1.01
± SE	0.43	12.6	6.8	0.21	0.22	8.7	6.8	0.23

* Volume of urine in ml/min.
** Sodium excretion in µEq/min.
† Potassium excretion in µEq/min.
†† Ratio of sodium over potassium excretion.

TABLE 27

Sodium and potassium excretion-

effect of posture.

LABILE HYPERKINETIC HYPERTENSION

NAME	RECUMBENT				UPRIGHT			
	VOLUME*	Na**	K†	Na/K††	VOLUME*	Na**	K†	Na/K††
J.G.	3.25	227	54	4.2	0.19	1	10	0.1
N.F.	3.56	260	68	3.82	0.57	46	39	1.18
A.C.	1.48	88	45	1.96	1.18	53	44	1.20
J.F.P.	2.16	162	34	4.76	1.26	54	33	1.64
P.D.	2.98	161	112	1.44	0.42	9	37	0.24
J.L.	3.34	240	80	3.0	0.58	55	39	1.41
L.L.	4.48	193	54	3.57	3.54	120	78	1.54
G.P.	3.52	239	78	3.06	1.79	90	43	2.09
A.H.	3.29	148	50	2.96	0.41	13	20	0.65
G.L.	0.76	48	31	1.51	0.36	1	8	0.13
C.L.	3.04	118	37	3.19	0.86	23	21	1.10
J.C.A.	4.06	190	63	3.02	2.14	40	51	0.78
F.L.	1.83	121	99	1.22	1.11	45	87	0.52
Mean	3.18	168.8	61.9	2.90	1.25	42.3	39.2	0.98
± SE	0.38	17.2	6.9	0.30	0.28	9.6	6.5	0.17

For explanation refer table 26.

TABLE 28

Statistical analysis of the effect of posture on sodium and potassium excretion.

Analysis of variance by randomized blocks.

CONTROL SUBJECTS

	Source of variation			
	SUBJECTS		POSTURE	
	F	p	F	p
SODIUM	0.58	N.S.	27.53	<0.01
POTASSIUM	1.99	N.S.	20.1	<0.01
Na/K RATIO	1.72	N.S.	11.77	<0.01

LABILE HYPERKINETIC HYPERTENSION

	Source of variation			
	SUBJECTS		POSTURE	
	F	p	F	p
	1.88	N.S.	51.80	<0.01
	2.89	<0.05	10.2	<0.01
	2.27	N.S.	50.75	<0.01

TABLE 29

Statistical analysis of the effect of posture on sodium and potassium excretion between the two groups.

	RECUMBENT		UPRIGHT	
	Unpaired Student t test			
	t	p	t	p
SODIUM	-1.65	N.S.	-0.31	N.S.
POTASSIUM	1.07	N.S.	0.29	N.S.
Na/K RATIO	-2.56	<0.02	0.15	N.S.

The observed differences between subjects disappeared in the upright position. The results of this study are summarized in table 30.

b - *Discussion*

Patients presented in this study are interesting from several points of view. They are relatively young with a mean age for the group of 27.7 years, which compares very well with the mean age of 23.5 years for the group of control subjects. It is remarkable, as evident from table 21, that the majority of patients had, at the time of the study, their mean blood pressure within or near to the normal limit (the higher limit of the "normal" range being 106 mmHg of mean blood pressure). This fact, together with postural tachycardia, is the principal characteristic of these patients and is in accordance with the observation of Widimský et al. (284), Eich et al. (61), and Frohlich et al. (80). The definition of this disease and the criteria used in the selection of our patients were discussed in part II-A-1 and part III-A-2.

The patients are also interesting by their high incidence of associated symptoms (table 31). The presence and the frequency of these symptoms have already been reported by our group (137). We may emphasize the frequency of gastrointestinal symptoms and ulcers also noted early by our group (136) contrasting with the observation of low incidence of duodenal ulceration in patients with stable essential hypertension reported by other workers (180). Some implications of this wide range of symptoms are considered in relation to possible disturbances of the cyclic AMP system in the general discussion.

Attention must be drawn to the fact that for the purpose of simplication only the means of mean blood pressure(diastolic plus one third of pulse pres-

TABLE 30

Summary of observations in the study of the effect of posture.

I CHANGES DURING UPRIGHT POSITION (AS COMPARED TO RECUMBENT) WITHIN EACH GROUP.

CONTROL SUBJECTS

PULSE	MBP	CREAT	VOLUME	cAMP	Na	K	Na/K
⇧	⇧	⇩	↓	⇩	↓	↓	↓

LABILE HYPERKINETIC HYPERTENSION

↑	—	⇩	↓	↑	↓	↓	↓

II DIFFERENCES OBSERVED IN LABILE HYPERKINETIC HYPERTENSION (AS COMPARED TO CONTROL SUBJECTS)

RECUMBENT

⇧	↑	—	—	—	⇧	⇩	↑

UPRIGHT

↑	↑	—	—	↑	—	—	—

— No difference observed; ⇧ insignificant difference; ↑ difference significant at the level of 95%; ↑ difference significant at the level of 99% (considered as significant, when established as such with at least one of the statistical tests used).

TABLE 31

Associated symptoms observed in patients with labile hyperkinetic hypertension

Total number of patients	24
Dermographism	17
Dyspepsia	8
Proven gastroduodenal ulcer	5
Neurovegetative pattern of glucose tolerance test*	7
Anxiety and/or depression	22

* Characterized by a steep increase over 160 mg% in the first 30 minutes of glucose tolerance test with the remaining part of the curve being normal.

sure) have been reported in this thesis, resulting in some loss of information, our interest being mainly centered on cyclic AMP data.

The decrease in creatinine and electrolytes excretion, accompanying postural antidiuresis is in accordance with recognized physiological adaptation to posture (64, 65). In this respect, the postural adaptation is similar in both groups of subjects studied. It is however interesting to note the higher sodium and lower potassium excretion in patients with labile hyperkinetic hypertension, leading to a significant increase of Na/K ratio. It was demonstrated that patients with stable essential hypertension show a greater and more rapid rejection of salt loads (83), our subjects, however, were maintained on a calculated diet and the study was performed when the equilibrium between electrolytes intake and excretion was reached. Our observation may therefore be specific to patients with labile hyperkinetic hypertension.

Cyclic AMP present in urine is known to have a double origin. A large part derives from plasma by glomerular filtration and about a third originates from kidney (23). Kaminsky et al. demonstrated a good correlation between urinary cyclic AMP and creatinine excretion in normal subjects (118). When precaution against "lability" with correlation tests are taken, it is possible to state that, in our study, a correlation between creatinine and cyclic AMP in the recumbent position is observed in control subjects, while this correlation in patients is poor (table 32). In the upright position, subjects react in an opposite way - the correlation loses its significance in control subjects and becomes significant in patients. We prefer to avoid any speculation on this finding. Unfortunately, the glomerular filtration rate was not measured exactly in this study and so, only the creatinine excretion is considered as an approximate indicator of kidney function, with all its known limitations (plasma creatinine values being within normal range in both groups) (11, 26).

TABLE 32

Correlation between creatinine and cyclic AMP excretions.

1 - STUDY OF POSTURE

	CONTROL SUBJECTS			PATIENTS		
	r	*t*	*p*	*r*	*t*	*p*
RECUMBENT	0.7418	3.49	<0.01	0.4916	1.96	N.S.
UPRIGHT	0.5670	2.14	N.S.	0.7575	4.02	<0.005

2 - STUDY OF THE EFFECT OF ISOPROTERENOL

	CONTROL SUBJECTS			PATIENTS		
RECUMBENT	0.8726	4.37	<0.005	0.2807	0.65	N.S.
ISOPROTE-RENOL	0.4940	1.39	N.S.	0.0875	0.19	N.S.

3 - STUDY OF THE EFFECT OF PROPRANOLOL IN PATIENTS

	PLACEBO			PROPRANOLOL		
RECUMBENT	0.6842	2.09	N.S.	0.8425	3.49	<0.02
UPRIGHT	0.4194	1.15	N.S.	0.6257	1.79	N.S.

The response to upright posture in cyclic AMP excretion, considered by the covariance or expressed per gram of creatinine, was significantly different in both groups of subjects under study. Cyclic AMP excretion decreased in control subjects while it increased in patients; possible reasons for this opposite reaction are considered in the general discussion.

2 - STUDY OF ISOPROTERENOL INFUSION

a - *Effect on pulse rate, blood pressure, excretion of cylic AMP and electrolytes.*

Individual data from this study are presented in tables 33 and 34 for control subjects and patients respectively. The two groups of subjects responded differently to a stimulation by isoproterenol. The clinical observations contrast somehow with objective measurements. Control subjects frequently complained of palpitations (in five of the eight cases) and even anxiety was a spontaneous complaint, especially during the first 30 minutes of infusion (in four of the eight cases). In some subjects, it was necessary to slow down the initial infusion rate for a few minutes, however all of them received the total dose within the four hour period. Only one out of seven patients complained of subjective adverse reactions and in one other case (P.D.) disorientation and behavioral changes during the eight hours following the end of infusion were observed. The latter reaction was fortunately only transitory. A peculiar observation was that two thirds of subjects from both groups were asleep after the first hour of infusion.

Isoproterenol infusion, increased the pulse rate in both groups, but the significant level of 99% was reached in patients only. Mean blood pressure (mean of all recordings during the four hour infusion) showed large variations

TABLE 33

Basic data and cyclic AMP excretion-

effect of isoproterenol.

CONTROL SUBJECTS

NAME	AGE	SEX	PR*		MBP**		VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	RECUMBENT cAMP/Cr 1
			30'	4hrs	30'	4hrs					
Y.N.	21	M	89	89	70	74	1.20	0.91	1.94	2.14	2.37
E.S.	21	M	101	98	64	80	3.17	1.33	2.86	2.15	2.54
P.S.	22	M	87	77	112	114	1.46	1.40	3.69	2.64	3.57
J.D.	22	F	72	74	66	63	4.30	1.58	3.81	2.04	5.17
P.B.	23	M	91	81	82	86	3.54	1.25	2.61	2.05	2.36
N.T.	24	F	92	89	59	60	3.33	1.17	2.23	1.90	4.00
Y.D.	24	M	82	97	100	113	5.17	1.90	4.07	2.14	3.21
A.S.	25	M	92	97	96	94	3.37	1.45	7.53	5.19	5.03
Mean			88.5	87.75	81.12	85.50	3.19	1.37	3.59	2.53	3.53
± SE			2.99	3.35	6.90	7.27	0.47	0.10	0.62	0.39	0.40

* Mean pulse rate per minute for the first 30' and for the complete 4 hour period of isoproterenol infusion.

** Mean of blood pressure in mmHg (diastolic + 1/3 of pulse pressure) for the first 30' and for the complete 4 hour period (refer method).

† Volume of urine in ml per minute during the four hour infusion period.

†† Creatinine excretion in µg/min. during the four hour infusion period.

§ Cyclic AMP excretion in nanomol/min.

§§ Cyclic AMP excretion expressed in µmol and corrected per g of creatinine.

1 Cyclic AMP excretion (in µmol/g of creatinine) in recumbent position without infusion, showed for direct comparison.

TABLE 34

Basic data and cyclic AMP excretion-

effect of isoproterenol.

LABILE HYPERKINETIC HYPERTENSION

NAME	AGE	SEX	PR*		MBP**		VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	RECUMBENT cAMP/Cr[1]
			30'	4hrs	30'	4hrs					
N.F.	15	M	90	97	74	72	3.42	1.43	4.45	3.10	2.89
J.P.P.	21	M	103	105	81	94	5.22	1.09	7.04	6.43	1.38
P.D.	22	M	83	93	82	84	3.39	1.53	6.53	4.27	4.18
J.L.	28	M	104	110	76	78	3.01	1.17	5.06	4.31	4.40
G.L.	37	F	84	92	83	79	2.89	0.89	5.68	6.34	4.77
C.L.	37	F	98	102	94	90	4.39	1.00	2.99	2.99	2.61
J.C.A.	39	M	117	115	106	101	5.70	1.31	3.48	2.65	2.03
Mean			97.00	102.00	85.14	85.43	4.01	1.20	5.03	4.30	3.18
± SE			4.63	3.26	4.23	3.82	0.42	0.09	0.57	0.59	0.49

For explanation refer table 33.

within the group of control subjects, but total changes were insignificant. Mean blood pressure in patients decreased primarily because of a diminution in diastolic pressure. The systolic pressure demonstrated a state of alertness in patients - various stimuli such as a visit of persons outside of the team or noises had frequently an immediate effect on the systolic blood pressure, increasing it by 50 to 100 mmHg.

The creatinine excretion increased significantly in control subjects while high variability with no significant change was observed in the group of patients (table 35). Cyclic AMP excretion showed an opposite response in control subjects and patients - decreasing significantly in the former and increasing significantly in the latter group (table 36). The degree of correlation between creatinine and cyclic AMP excretions is highly lowered by isoproterenol infusion (table 32).

When the intergroup differences of the reaction to isoproterenol infusion are considered, a significant increase of pulse rate and cyclic AMP excretion, as compared to control subjects, is observed (table 37). The "amplitude of change" due to isoproterenol infusion is significantly different between the two groups of subjects in cyclic AMP excretion (table 38).

Individual data of the effect of isoproterenol infusion on the excretion of sodium and potassium is presented in table 39 for both groups of subjects. No change in sodium excretion was observed in either of the two groups (as compared to corresponding data in recumbent position without infusion). In contrast, potassium excretion decreased in both groups: this change was significant in control subjects only and resulted in an increase of the sodium/potassium ratio (table 40). When the intergroup differences of sodium, potassium and creatinine excretion are considered, no evident change is observed (table 41).

TABLE 35

Statistical analysis of the effect of isoproterenol infusion on basic data *

Analysis of variance by randomized blocks.

CONTROL SUBJECTS

	Source of variation			
	SUBJECTS		ISOPROTERENOL	
	F	p	F	p
PULSE RATE	1.39	N.S.	4.72	N.S.
MEAN BLOOD PRESSURE	4.06	<0.01	0.03	N.S.
VOLUME	3.89	<0.05	1.37	N.S.
CREATININE EXCRETION	1.44	N.S.	15.14	<0.01

LABILE HYPERKINETIC HYPERTENSION

Source of variation			
SUBJECTS		ISOPROTERENOL	
F	p	F	p
0.75	N.S.	16.58	<0.01
0.63	N.S.	3.56	N.S.
1.5	N.S.	3.28	N.S.
9.08	<0.01	0.0	N.S.

TABLE 36

Statistical analysis of the effect of isoproterenol infusion on cyclic AMP excretion *

CONTROL SUBJECTS — LABILE HYPERKINETIC HYPERTENSION

Analysis of covariance (cyclic AMP per minute, reduced for creatinine excretion)

F	p	F	p
5.99	<0.05	2.71	N.S.

Paired Student t test (cyclic AMP per g of creatinine)

t	p	t	p
2.56	<0.05	-1.63	N.S.

Wilcoxon matched-paris test (cyclic AMP per g of creatinine)

T	p	T	p
1	<0.05	2	<0.05

* As compared to corresponding data in recumbent position without infusion in respective subjects.

TABLE 37

Statistical analysis of the effect of isoproterenol infusion on basic data and cyclic AMP excretion between the two groups.

Unpaired Student *t* test

	RECUMBENT		ISUPREL	
	t	*p*	*t*	*p*
PULSE RATE	0.31	N.S.	-3.02	<0.01
MEAN BLOOD PRESSURE	-1.78	N.S.	0.01	N.S.
VOLUME	-0.18	N.S.	-1.28	N.S.
CREATININE EXCRETION	0.40	N.S.	1.23	N.S.
CYCLIC AMP (PERg CREAT)	-0.59	N.S.	-2.56	<0.05

Analysis of covariance (cyclic AMP per minute, reduced for creatinine excretion)

F	*p*
-0.06	N.S.

F	*p*
4.18	N.S.

Wilcoxon test for two samples (cyclic AMP per g of creatinine)

T	*p*
68	N.S.

T	*p*
41	<0.01

TABLE 38

Statistical analysis of changes of basic data and cyclic AMP excretion by isoproterenol infusion (on data modified to eliminate negative values).

PULSE RATE*	Mean	S^2	$t^\dagger$	p
CONTROL versus	90.00	169.43	1.88	N.S.
PATIENTS	75.86	256.81		

MEAN BLOOD PRESSURE**	Mean	S^2	$t^\dagger$	p
CONTROL versus	101.00	231.43	-1.67	N.S.
PATIENTS	114.43	251.61		

CYCLIC AMP§	Mean	S^2	$t^\dagger$	p
CONTROL versus	10.999	1.223	2.59	<0.05
PATIENTS	8.883	3.925		
			$T^{\dagger\dagger}$	p
			90	≤0.01

For explanations refer table 25

TABLE 39

Sodium and potassium excretion- effect of isoproterenol infusion

CONTROL SUBJECTS

NAME	VOLUME*	Na**	K†	Na/K††
J.N.	1.21	63	25	2.52
E.S.	3.16	212	29	7.31
P.S.	1.46	117	54	2.17
J.D.	4.29	77	17	4.53
P.B.	3.54	174	57	3.05
Y.D.	5.17	186	25	7.44
A.S.	3.37	88	40	2.2
Mean	3.19	131.0	35.2	4.17
± SE	0.47	22.4	5.8	0.88
MEAN[1]	2.74	131.7	76.0	1.84
± SE	0.37	16.9	8.8	0.29

LABILE HYPERKINETIC HYPERTENSION

NAME	VOLUME*	Na**	K†	Na/K††
N.F.	3.42	113	82	1.38
J.P.P.	5.33	230	63	3.65
P.D.	3.39	102	41	2.49
J.L.	3.01	226	39	5.79
G.L.	2.89	58	15	3.87
C.L.	4.39	183	13	14.08
J.C.A.	5.70	120	29	4.14
Mean	4.00	147.4	40.3	5.05
± SE	0.42	25.0	9.5	1.59
Mean[1]	2.84	168.4	60.7	2.96
± SE	0.41	27.2	11.1	0.44

For explanations refer table 26.

1 Mean and standard errors of data obtained in recumbent position without infusion in respective subjects (for individual data refer tables 26 and 27).

TABLE 40

Statistical analysis of the effect of isoproterenol infusion on sodium and potassium excretion.

Analysis of variance by randomized blocks.

CONTROL SUBJECTS

	Source of variation			
	SUBJECTS		ISOPROTERENOL	
	F	p	F	p
SODIUM	1.54	N.S.	0.04	N.S.
POTASSIUM	1.19	N.S.	14.14	<0.01
Na/K RATIO	0.91	N.S.	5.61	N.S.

LABILE HYPERKINETIC HYPERTENSION

Source of variation			
SUBJECTS		ISOPROTERENOL	
F	p	F	p
2.18	N.S.	0.51	N.S.
1.63	N.S.	2.57	N.S.
1.07	N.S.	1.65	N.S.

TABLE 41

Statistical analysis of the effect of isoproterenol infusion on sodium and potassium excretion between the two groups.

Unpaired Student *t* test

	t	p
SODIUM	-0.48	N.S.
POTASSIUM	-0.44	N.S.
Na/K RATIO	-0.48	N.S.

The summary of all the above observations is presented in table 42.

b - *Discussion*

Isoproterenol infusion in the study of Frohlich et al. induced a rise of cardiac index and ventricular ejection rate in patients with labile hypertension more markedly than in patients with stable hypertension (80). In our experience, a higher increase of heart rate was obtained in patients with labile hyperkinetic hypertension, than in control subjects.

The effect of isoproterenol, a β-adrenergic agonist, was shown to be mediated by cyclic AMP. This is supported by the fact that isoproterenol increased the accumulation of cyclic AMP in aortic tissue (272) and exogenous cyclic AMP mimicked the effect of isoproterenol in smooth msucle (148).Volicer and Hynie demonstrated a direct relationship between vasodilatation induced by isoproterenol and a simultaneous increase in the formation of cyclic AMP (272). Kaminsky et al. reported an increase in plasma level of cyclic AMP during the infusion of isoproterenol in human (116). In addition, they stated that this β-adrenergic stimulation leads to a reduction of nephrogenous cyclic AMP, resulting in urinary levels which do not correspond to those reached in the plasma. Our results in control subjects are in accordance with this observation. In our study, a real decrease in cyclic AMP excretion during isoproterenol infusion in control subjects, as compared to the excretion in recumbent position was observed. In contrast to this finding, the excretion of cyclic AMP was increased by isoproterenol infusion in patients with labile hyperkinetic hypertension. Thus, the behavior of the two groups in their response in the excretion of cyclic AMP during isoproterenol infusion is similar to the reaction in upright position; possible reasons for this opposite response are

TABLE 42

Summary of observations in study of the effect of isoproterenol infusion.

I CHANGES INDUCED BY ISOPROTERENOL INFUSION (AS COMPARED TO RECUMBENT (POSITION)

CONTROL SUBJECTS

PULSE	MBP	CREAT	VOLUME	cAMP	NA	K	Na/K
⇧	—	▲	⇧	⇩	—	▼	⇧

LABILE HYPERKINETIC HYPERTENSION

▲	⇩	—	⇧	▲	—	⇩	⇧

II DIFFERENCES INDUCED BY ISOPROTERENOL INFUSION IN PATIENTS WITH LABILE HYPERKINETIC HYPERTENSION (AS COMPARED TO CONTROL SUBJECTS)

▲	—	⇩	⇧	▲	—	—	—

— No difference observed.

⇧ Insignificant difference; ▲ difference significant at the level of 95%.

▲ Difference significant at the level of 99% (considered as significant when established as such with at least one of the statistical tests used).

considered in the general discussion. Comparison of the effect of upright posture and isoproterenol infusion in the two groups of subjects are made in fig. 9.

The effect of isoproterenol infusion on different renal parameters has been extensively studied. Gill and Casper recently,after infusing isoproterenol into the renal artery, demonstrated the increase of the urinary volume and the free water clearance in hypophysectomized dogs (87). A similar effect on urinary volume was observed in our human subjects. In an extensive study in humans, Meurer observed also that β-adrenergic stimulation induced an increase of the urinary volume (184). Furthermore he noted a decrease in the clearance of potassium beginning at the end of the first hour of infusion. Our results are also confirmatory in this respect. Induction of hypokalemia by adrenergic stimulation was demonstrated nearly forty years ago (173). Beta-adrenergic receptors may play a part in this mechanism since Massara et al. abolished the epinephrine-induced decrease in plasma potassium in man by a pretreatment with propranolol (173). The explanation for the changes in plasma and urinary potassium are missing, but some interrelation with the release and metabolism of cyclic AMP was evoked (50, 221) (refer part II-B-7-c).

3 - STUDY OF PROPRANOLOL ADMINISTRATION

a - *Effect on pulse rate, blood pressure, excretion of cyclic AMP and electrolytes.*

This study was conducted with seven out of a total of fourteen patients with labile hyperkinetic hypertension. Individual data of pulse rate, blood pressure, cyclic AMP and creatinine excretion are presented in tables 43 and 44 for periods during the administration of placebo and propranolol respec-

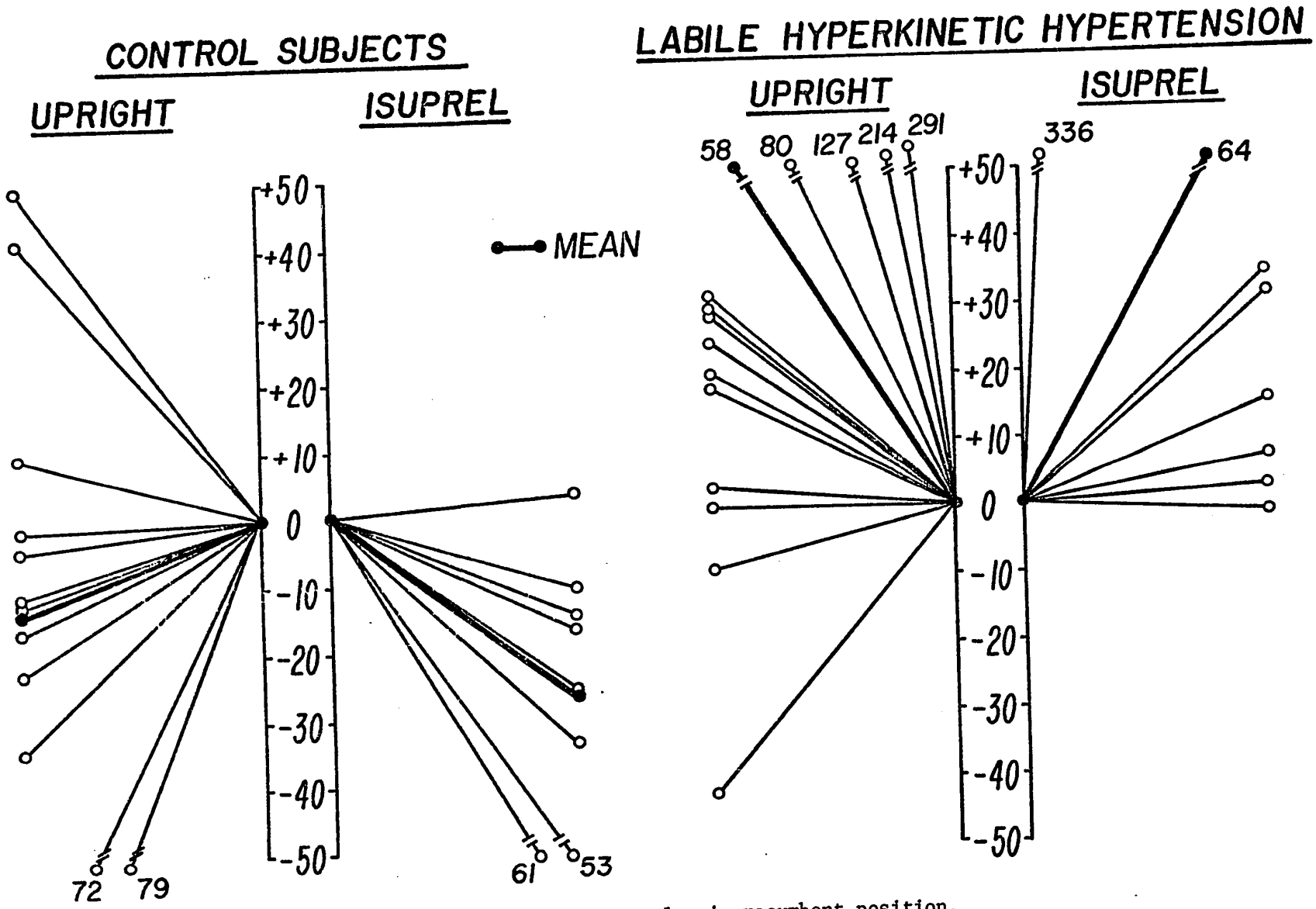


Fig. 9 Percentage of change of cyclic AMP excretion from value in recumbent position.

TABLE 43

Basic data and cyclic AMP excretion
effect of posture and placebo administration

LABILE HYPERKINETIC HYPERTENSION (ON PLACEBO*)

NAME	AGE	SEX	RECUMBENT						UPRIGHT					
			PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§
N.F.	15	M	81	96	3.56	1.41	3.89	2.90	93	73	0.57	1.24	4.46	3.60
A.C.	21	M	81	96	1.48	0.90	1.96	2.16	93	104	1.18	0.96	2.46	2.55
P.D.	22	M	58	92	2.98	1.31	5.48	4.17	85	83	0.42	0.66	3.59	5.47
J.L.	28	F	78	97	3.24	1.37	6.03	4.41	92	101	0.58	1.14	6.51	5.69
G.P.	30	M	69	104	3.52	2.08	5.48	2.64	96	112	1.79	0.61	5.06	8.26
A.H.	30	M	67	100	3.29	1.14	1.32	1.16	76	101	0.41	0.60	2.72	4.53
C.L.	37	F	77	93	3.04	0.95	2.49	2.61	78	88	0.86	0.69	2.17	3.12
Mean			73.00	96.86	3.03	1.31	3.81	2.86	87.17	94.57	0.83	0.84	3.85	4.75
± SE			3.26	1.55	0.27	0.15	0.72	0.42	3.01	5.16	0.19	0.10	0.59	0.73

LABILE HYPERKINETIC HYPERTENSION (NO MEDICATION*)

J.P.P.	21	M	84	106	2.16	1.27	1.76	1.38	116	95	1.26	1.00	3.16	3.13
P.C.	27	M	68	108	6.70	1.41	2.67	1.88	96	121	3.06	1.22	2.30	1.88
L.L.	29	F	68	121	4.48	0.94	4.94	5.25	84	111	3.54	2.05	9.82	4.78
G.L.	37	F	97	121	0.76	0.82	3.91	4.77	97	135	0.36	0.59	3.64	6.15
J.C.A.	39	M	70	94	4.06	1.30	2.64	2.03	81	96	2.14	1.24	4.53	3.65
F.L.	41	M	84	109	1.83	1.83	7.13	3.89	91	103	1.11	1.74	6.97	4.01
Mean			78.5	109.83	3.33	1.26	3.84	3.20	94.17	110.17	1.91	1.31	5.07	3.93
± SE			4.82	4.16	0.88	0.15	0.80	0.67	5.08	6.37	0.50	0.21	1.15	0.59

For explanations refer table 20.

* Same data as in table 21.

TABLE 44

Basic data and cyclic AMP excretion-
effect of posture during propranolol administration in labile hyperkinetic hypertension.

NAME	AGE	SEX	RECUMBENT						UPRIGHT					
			PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§	PR*	MBP**	VOLUME†	CREAT††	cAMP/min§	cAMP/Cr§§
N.F.	15	M	64	71	4.21	1.26	5.55	4.40	91	87	0.77	1.24	5.60	4.51
A.C.	21	M	64	85	2.67	1.17	2.41	2.05	79	76	0.86	1.03	2.00	1.94
P.D.	22	M	62	89	3.69	1.33	4.14	3.12	102	90	0.27	1.20	3.98	3.30
J.L.	28	M	77	110	4.35	1.39	5.36	3.85	84	85	2.01	0.90	3.44	3.81
A.H.	30	M	56	85	1.47	1.17	1.82	1.55	53	94	0.37	1.07	0.55	0.52
G.P.	30	M	81	73	4.50	1.98	7.18	3.62	65	93	1.72	1.76	5.44	3.12
C.L.	37	F	63	81	4.17	1.00	2.30	2.28	66	85	3.54	1.14	2.73	2.38
Mean			66.71	84.86	3.58	1.33	4.11	2.98	77.14	87.14	1.36	1.19	3.39	2.80
± SE			3.4	4.9	0.42	0.12	0.76	0.39	6.4	2.3	0.44	0.10	0.69	0.50

For explanations refer table 20.

tively. Table 43 shows in addition results in patients receiving no medication whatsoever to permit consideration of possible effects of placebo. The first statistical analysis was performed to determined the latter effect. Results presented in table 45 demonstrate that placebo had no effect, the only exception being a difference in the mean blood pressure in recumbent position between groups receiving no medication and placebo. The total analysis of variance clearly indicates (table 46) that there is a significant difference in cyclic AMP excretion between the three groups, i.e. receiving no medication, placebo and propranolol. The Newman-Keuls test was performed on these data in spite of the fact that the "no medication" group included six subjects which were different from seven others included in the groups of "placebo" and "propranolol". A modified Newman-Keuls test for unequal groups was therefore used (286). The results show that cyclic AMP excretion is different ($p < 0.05$) during the administration of propranolol and that there is no difference between "no medication" and "placebo" groups.

The reaction to upright position is characterized by a highly significant rise of pulse rate in the group receiving the placebo, while it was practically nonexistent in the group receiving propranolol (table 47). The excretion of cyclic AMP (table 48) was significantly increased by upright position in patients when receiving placebo and again, this increase was abolished by the administration of propranolol.

When an analysis is performed between the data from periods of placebo and propranolol administration in the two respective positions (table 49), it is evident that the mean blood pressure is significantly different in the recumbent position, while the excretion of cyclic AMP and creatinine change only

TABLE 45

Statistical analysis of the effect of placebo on different parameters (as compared to patients with no medication)

Unpaired Student t test

	RECUMBENT		UPRIGHT	
	t	p	t	p
PULSE RATE	0.96	N.S.	1.15	N.S.
MEAN BLOOD PRESSURE	3.09	<0.02	1.92	N.S.
VOLUME	0.34	N.S.	2.13	N.S.
CREATININE EXCRETION	-0.21	N.S.	2.06	N.S.
CYCLIC AMP (PER g CREAT)	0.58	N.S.	0.84	N.S.

TABLE 46

Analysis of variance of the effect of no medication, placebo and propranolol in patients with labile hyperkinetic hypertension on cyclic AMP excretion.

SOURCE OF VARIATION	d.f.	M.S.	F	p
DIFFERENT TREATMENT	2	7.5325	4.55	<0.05
ERROR	17	1.6577		

Newman-Keuls test: "No medication" and "Placebo" do not differ from each other; "Propranolol" differs from both with the value of the $F = 6.13$, critical value for $1-\alpha$ 0.95 4.45, $1-\alpha$ 0.99 8.33 i.e. $p < 0.05$

TABLE 47

Statistical analysis of the effect of posture on basic data during the administration of different medication in patients with labile hyperkinetic hypertension

Analysis of variance by randomized blocks.

PLACEBO

	Source of variation			
	SUBJECTS		POSTURE	
	F	p	F	p
PULSE RATE	2.08	N.S.	16.58	<0.01
MEAN BLOOD PRESSURE	2.28	N.S.	0.3	N.S.
VOLUME	0.72	N.S.	37.79	<0.01
CREATININE EXCRETION	0.79	N.S.	5.92	N.S.

PROPRANOLOL

Source of variation			
SUBJECTS		POSTURE	
F	p	F	p
1.07	N.S.	2.52	N.S.
0.71	N.S.	0.18	N.S.
3.31	N.S.	28.58	<0.01
8.30	<0.01	4.09	N.S.

TABLE 48

Statistical analysis of the effect of posture on cyclic AMP excretion during the administration of different medication in patients with labile hyperkinetic hypertension.

PLACEBO PROPRANOLOL

Analysis of covariance (cyclic AMP per minute, reduced for creatinine excretion)

F	p
2.34	N.S.

F	p
0.01	N.S.

Paired Student *t* test (cyclic AMP per g of creatinine)

t	p
-2.58	<0.05

t	p
--0.73	N.S.

Wilcoxon matched-pairs test (cyclic AMP per g of creatinine)

T	p
0	<0.01

T	p
10	N.S.

TABLE 49

Statistical analysis of the effect of propranolol administration (as compared to placebo) on basic data and cyclic AMP excretion

Unpaired Student *t* test

	RECUMBENT		UPRIGHT	
	t	*p*	*t*	*p*
PULSE RATE	1.34	N.S.	1.47	N.S.
MEAN BLOOD PRESSURE	2.35	<0.05	1.31	N.S.
VOLUME	-1.09	N.S.	-1.12	N.S.
CREATININE	-1.10	N.S.	-2.41	<0.05
CYCLIC AMP (PER g CREAT)	-0.20	N.S.	2.20	<0.05

Wilcoxon test for two samples (cyclic AMP per g of creatinine)

T	*p*
15	N.S.

T	*p*
3	N.S.

in the upright position. The "amplitude of change" due to upright position (table 50) is significantly different between "placebo" and "propranolol" periods for the cyclic AMP excretion only.

Individual data of sodium and potassium excretion, as well as their ratio in both positions during propranolol administration, are presented in table 51. The excretion of sodium and potassium decreased highly significantly in the upright position, independently of what the patients received either the placebo or the propranolol; the decrease in sodium excretion being more important, the sodium/potassium ratio decreased highly significantly (table 52). When the excretion of electrolytes in each position is compared between periods of "placebo" and "propranolol", no significant change is found, but a small increase in sodium and potassium in both positions is observed (table 53).

The summary of the above observations is presented in table 54.

b - *Discussion*

The β-blocking effect of propranolol is well established, particularly that of its L-form; it was active in antagonizing the response to isoproterenol infusion in man (22) and in relief of anxiety (92, 20). This agent was also able to reduce gastric secretion and the frequency of stress-induced gastric ulcer in rats (193) and to reduce the rise of systolic blood pressure during human coitus (76). Propranolol showed no effect on blood pressure in intact, unstimulated animals, but caused a reduction of catecholamine content of the rat heart (175). Its usefulness was demonstrated in the treatment of

TABLE 50

Statistical analysis of changes in basic data and cyclic AMP excretion in response to upright posture (on modified data to eliminate negative values)

PULSE RATE*	Mean	S^2	$t^\dagger$	p
PLACEBO versus	85.42	89.61	-0.52	N.S.
PROPRANOLOL	89.57	352.61		

MEAN BLOOD PRESSURE**	Mean	S^2	$t^\dagger$	p
PLACEBO versus	102.28	123.90	0.63	N.S.
PROPRANOLOL	97.71	237.23		

CYCLIC AMP§	Mean	S^2	$t^\dagger$	p
PLACEBO versus	8.11	3.73	-2.76	<0.02
PROPRANOLOL	10.18	0.19		

$T^{\dagger\dagger}$	p
0	<0.05

For explanations refer table 25.

TABLE 51

Sodium and potassium excretion-

effect of posture during propranolol administration in labile hyperkinetic hypertension.

NAME	RECUMBENT				UPRIGHT			
	VOLUME*	Na**	K†	Na/K††	VOLUME*	Na**	K†	Na/K††
N.F.	4.21	253	101	2.50	0.77	55	85	0.65
A.C.	2.67	147	61	2.41	0.86	65	52	1.25
P.D.	3.69	173	78	2.22	0.27	4	25	0.16
J.L.	4.35	296	100	2.96	2.01	71	50	1.42
A.H.	1.47	72	34	2.12	0.37	18	33	0.55
G.P.	4.50	306	95	3.22	1.72	116	67	1.73
C.L.	4.16	142	50	2.84	3.54	57	21	2.71
Mean	3.58	198.4	74.1	2.61	1.36	55.1	47.6	1.21
± SE	0.42	33.3	10.0	0.15	0.44	13.8	8.8	0.32
Mean[1]	3.03	179.1	67.1	2.77	0.83	41.3	34.7	1.21
± SE[1]	0.27	25.4	9.7	0.30	0.19	10.8	3.8	0.24

For explanations refer table 26.

1 Mean and standard error of data from eight patients receiving the placebo during the study of posture (for individual data refer table 27).

TABLE 52

Statistical analysis of the effect of posture on sodium and potassium excretion during the administration of different medications in patients with labile hyperkinetic hypertension

Analysis of variance by randomized blocks.

PLACEBO

	Source of variation			
	SUBJECTS		POSTURE	
	F	p	F	p
SODIUM	2.09	N.S.	38.43	<0.01
POTASSIUM	1.9	N.S.	13.95	<0.01
Na/K RATIO	4.79	<0.05	47.1	<0.01

PROPRANOLOL

Source of variation			
SUBJECTS		POSTURE	
F	p	F	p
2.95	N.S.	36.39	<0.01
5.39	<0.05	14.85	<0.01
3.575	N.S.	40.62	<0.01

TABLE 53

Statistical analysis of the effect of posture on sodium and potassium excretion between periods of placebo and propranolol administration in patients with labile hyperkinetic hypertension

Unpaired Student *t* test

	t	p
SODIUM	-0.46	N.S.
POTASSIUM	-0.50	N.S.
Na/K RATIO	0.48	N.S.

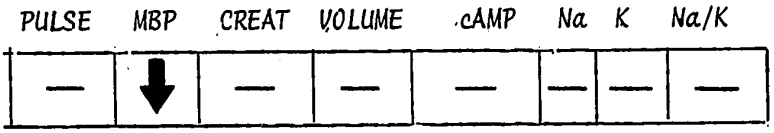
t	p
-1.07	N.S.
-1.34	N.S.
0.01	N.S.

TABLE 54

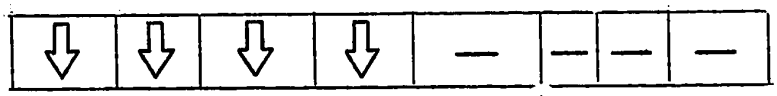
Summary of observations in study of the effect of propranolol

I PLACEBO EFFECT (AS COMPARED TO PATIENTS RECEIVING NO MEDICATION)

RECUMBENT



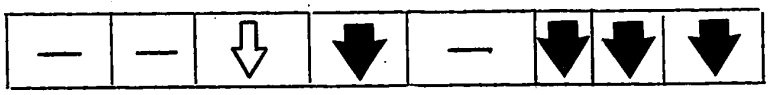
UPRIGHT



II CHANGES DURING UPRIGHT POSITION (AS COMPARED TO RECUMBENT) DURING THE ADMINISTRATION OF THE TWO MEDICATIONS

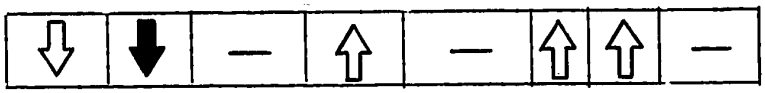
PLACEBO

PROPRANOLOL

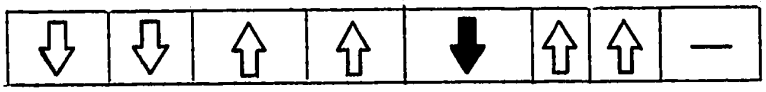


III DIFFERENCES OBSERVED DURING PROPRANOLOL ADMINISTRATION (AS COMPARED TO PLACEBO)

RECUMBENT



UPRIGHT



For explanations refer table 42.

hyperkinetic heart syndrome, idiopathic hypertrophic subaortic stenosis, and systemic hypertension (227). Its effect in the reduction of high blood pressure is paradoxical if its capacity to rise the peripheral resistance is considered (81), but Frohlich was able to demonstrate that its capacity to reduce the mean blood pressure is directly related to the height of resting pretreatment cardiac output (80). The capacity to reduce cardiac index in patients with borderline hypertension was demonstrated by Conway with a single intravenous dose of propranolol (47).

The present study confirms the efficiency of propranolol treatment in reducing the circulatory hyperkinesis in patients, in addition to its effects on a biochemical parameter, cyclic AMP excretion. This efficiency becomes evident when statistical results presented in tables 22, 23, 24, 25 and 47, 48, 49, 50 are compared. It is obvious from this comparison that the treatment with propranolol renders patients with labile hyperkinetic hypertension comparable to control subjects in the following respects: 1) pulse rate is highly significantly increased in patients whether on placebo or without medication; this characteristic is absent in both control subjects and patients during propranolol administration; 2) blood pressure is significantly elevated in patients with placebo or without any medication as compared to control subjects or patients during propranolol administration; 3) cyclic AMP excretion is significantly increased in untreated patients and this characteristic is also absent or abolished in control subjects or patients receiving propranolol. The "amplitude of change" of cyclic AMP was as significantly different between patients during the "propranolol" compared to the "placebo" period, as it was in control subjects compared to patients. Finally, propranolol administration

renders the correlation between creatinine and cyclic AMP excretion as significant in patients as it was in control subjects (refer table 32).

A graphical comparison of effects of upright posture on cyclic AMP excretion in patients during placebo and propranolol administration is presented in fig. 10.

C - CYCLIC AMP MEASUREMENT BY PROTEIN BINDING ASSAY

1 - EFFECT OF POSTURE AND ISOPROTERENOL INFUSION

Individual data of cyclic AMP excretion in the two groups of subjects studied in both positions and during isoproterenol infusion are presented in table 55. The statistical analysis of these data (table 56) shows that the only statistically significant change is induced by isoproterenol infusion: it lowers cyclic AMP excretion in control subjects while it causes an increase in the nucleotide excretion in labile hyperkinetic hypertension.

2 - DISCUSSION

This part of the study differs from the preceeding one only by the method used, since urinary samples were identical to those measured by the radioimmunoassay. The protein binding method was demonstrated in our hands to be presently the most reliable method of cyclic AMP measurement (refer tables 14 and 15). The results concerning the effect of posture are however different; the increase of cyclic AMP excretion in patients is still present, but the excretion of the nucleotide tends to be higher in the upright position also in control subjects.

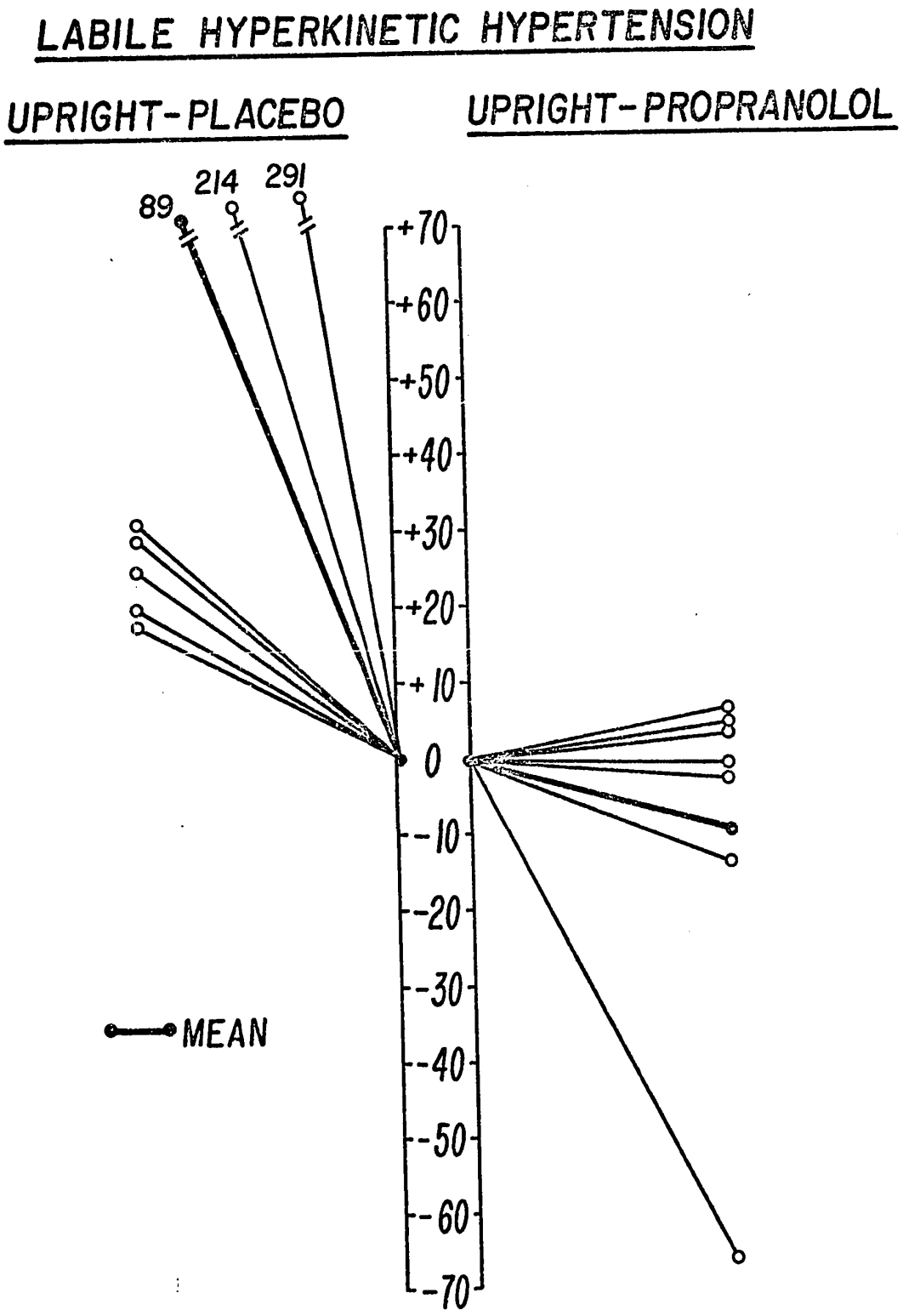


Fig. 10 Percentage of change of cyclic AMP excretion from values observed in recumbent position in the same patients receiving either the placebo or the propranolol.

TABLE 55

Excretion of cyclic AMP as measured by protein binding assay

CONTROL SUBJECTS

	RECUMBENT		UPRIGHT		ISOPROTERENOL	
NAME	cAMP/min[§]	cAMP/Cr[§§]	cAMP/min[§]	cAMP/Cr[§§]	cAMP/min[§]	cAMP/Cr[§§]
P.S.	5.27	4.03	4.53	4.21	4.31	2.88
J.D.	4.00	5.28	3.97	6.14	5.46	3.45
Y.D.	5.25	4.23	4.18	3.33	3.67	1.93
N.T.	2.28	2.75	2.39	3.69	2.07	1.76
A.S.	10.45	4.40	7.16	4.31	5.68	3.92
Mean	5.27	4.03	4.53	4.20	4.31	2.88
± SE	1.38	0.43	0.78	0.51	0.66	0.43

LABILE HYPERKINETIC HYPERTENSION

N.F.	4.84	3.44	4.77	3.69	5.33	3.72
J.P.P.	2.50	1.89	4.33	4.13	4.70	4.29
J.L.	6.06	4.25	5.55	4.65	5.97	5.08
C.L.	3.02	3.17	2.77	3.98	3.90	3.90
G.L.	4.45	5.21	3.65	5.92	5.70	6.37
J.C.A.	4.00	2.96	2.45	1.89	4.37	3.33
Mean	4.15	3.49	3.92	4.04	4.99	4.45
± SE	0.52	0.46	0.49	0.54	0.33	0.45

§ Cyclic AMP excretion in nmol/min.

§§ Cyclic AMP excretion in µmol/g of creatinine

TABLE 56

Statistical analysis of the effect of posture and isoproterenol on cyclic AMP excretion

Paired Student t test within two groups of subjects

	UPRIGHT		ISOPROTERENOL	
	t	p	t	p
CONTROL SUBJECTS	-0.52	N.S.	2.85	<0.05
LABILE HYPERKINETIC HYPERTENSION	-1.28	N.S.	-3.04	<0.05

Unpaired Student t test between two groups of subjects

	RECUMBENT	UPRIGHT	ISOPROTERENOL
t	0.84	0.2	-2.46
p	N.S.	N.S.	<0.05

The results of cyclic AMP excretion measured by the protein binding are slightly higher than the results obtained by the radioimmunoassay. A possible explanation for this difference is that our urine samples (originally 5 ml) became concentrated with repeated freezing and thawing - some of them having been measured repetitively by all three methods used within a period of two and half years. It is therefore important to emphasize that the stimulation of isoproterenol (urine samples thawed infrequently) leads to the same conclusion as drawn from the study using radioimmunoassay.

V - GENERAL DISCUSSION

The work described in this thesis was part of a larger study of the group of Genest on the relationship between the adrenergic nervous system and renin in labile hyperkinetic hypertension. The study was realized in collaboration with Drs Kuchel, Cuche, Boucher, Barbeau and Genest (137). The experimental design, in the previously reported and present studies, was similar in most aspects. A part of patients and control subjects were also identical for these two works. The general discussion of the present results must therefore be introduced by some observations from this reported study. Patients under the latter investigation presented hypertension over 140/90 mmHg, but their blood pressure decreased with physical and emotional rest. One of the main clinical characteristic of their hyperkinetic circulation was tachycardia accentuated by upright position and cold-pressure test. Hemodynamic studies*

*Hemodynamic studies were performed by Dr G. Dagenais, Hotel-Dieu Hospital.

performed in half of the patients showed an elevated cardiac index (4.3 ± SE 0.39 1/min./m^2) accompanied by normal or low peripheral resistance. These characteristics agree with observations of similar patients reported by other groups (61, 284, 80).

The above patients with labile essential hypertension, compared to control subjects, presented a significantly higher response in plasma renin activity to upright posture. Urinary catecholamines showed a significant increase in the excretion of epinephrine and norepinephrine in recumbent position (when compared to control subjects) with a further increase in response to upright posture. The urinary dopamine excretion in patients was significantly lower when related to norepinephrine. In response to β-adrenergic stimulation by isoproterenol, the patients presented an excessive and prolonged increase in plasma renin activity. It may be therefore concluded that these patients are characterized by qualitative and quantitative differences in catecholamines excretion as well as by an increased responsiveness of the renin-angiotensin system to several stimuli.

High plasma renin activity in patients with labile hypertension, as compared to normal subjects or patients with mild essential hypertension, has also been reported by another group (80). The β-adrenergic stimulation of renin release was demonstrated *in vitro* and *in vivo* (185, 286). An indirect hemodynamic as well as a direct sympathetic regulation of renin release was recently confirmed by Reid et al. (215).

Adrenergic receptors in patients suffering from essential hypertension were shown to have increased responsiveness to stimulation in several studies

of the groups of Goldenberg, Raab, Judson, Doyle, Mendlowitz and others (123). A similar increased responsiveness to angiotensin infusion in labile and stable essential hypertension was demonstrated by Kaplan and Silah (120). There is no doubt, at the present time, that adrenergic receptors or at least their β-component are part of adenyl cyclase (220). The results presented in this thesis may therefore be analyzed in relation to the above mentioned facts.

No differences in the baseline values (i.e. in recumbent position) for the excretion of cyclic AMP in patients with labile hyperkinetic hypertension were observed, but after the action of two stimuli, differences became evident. The first one was a mild physiological stimulus, assuming of the upright position. The two groups studied showed opposite responses: a slight decrease of the nucleotide excretion in control subjects contrasted with an increase in patients. The second stimulus used was of pharmacological nature, namely isoproterenol infusion. Here again the response of the two groups was contrasting and more pronounced. Graphical summary of our observation is presented in fig. 11.

These results leave little doubt that differences exist between the groups of subjects studied; their interpretation is difficult however at the present time. First of all, it is impossible to localize the origin of differences in cyclic AMP excretion without the possibility of evaluating the nephrogenous component of this excretion. The only way to achieve this would involve knowledge of the plasma levels of cyclic AMP; however, these measurements have not been completed at the time of writing this thesis.

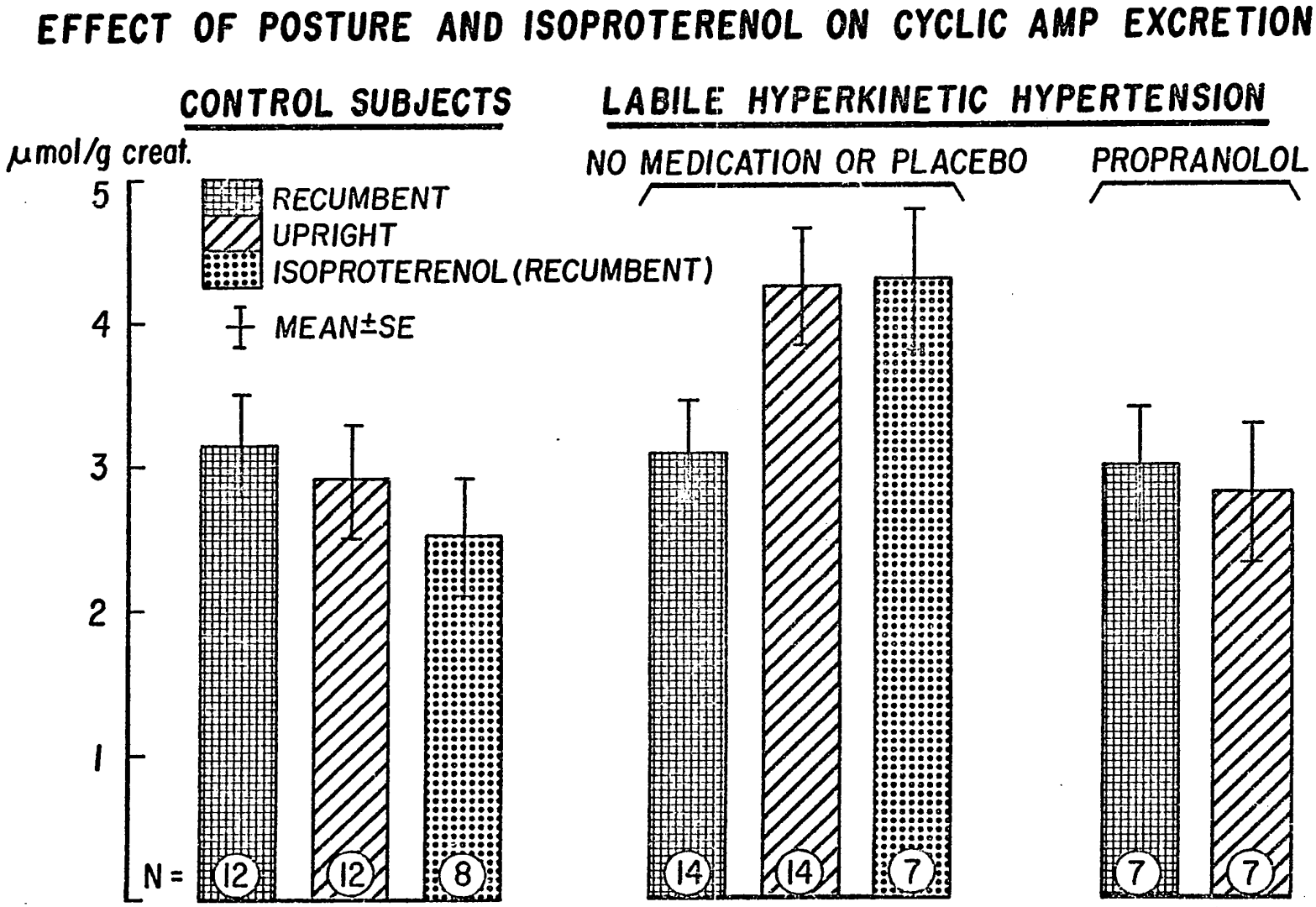


Fig.11 Excretion of cyclic AMP in µmol/g of creatinine as measured by radioimmunoassay. The effect of the two positions and isoproterenol infusion is demonstrated in control subjects and patients receiving no medication or placebo. Normalization of the effect of posture on cyclic AMP excretion is evident in the same patients after propranolol administration.

Some possible explanations can be proposed. 1) The observed *decrease* of cyclic AMP excretion in *control subjects*, especially evident after isoproterenol infusion, contrasting with the reported increase in plasma level (116), may be due to a decrease of the nephrogenous component of urinary cyclic AMP. This possibility has already been raised by Kaminsky in a similar study with normal subjects (116). The degree of decrease of cyclic AMP excretion, after prolonged isoproterenol stimulation in our study, is however so important in some of the subjects, that the possibility of tubular reabsorption must also be considered. The absence of tubular reabsorption of cyclic AMP was reported (23), but no study was performed under conditions of physiological or pharmacological stimulation. 2) The observed *increase* in cyclic AMP excretion during upright position and during isoproterenol infusion in *patients* (as compared to the decrease in control subjects) characterizes their opposite response to the same stimulation. These differences in response may be secondary to a disturbance in the mechanism regulating the excretion of cyclic AMP, or, rather in a more general sense, to a disturbance in the reactivity of adenyl cyclase in response to a hormonal stimulation, or, to a change in the metabolic fate of cyclic AMP in patients with labile hyperkinetic hypertension.

Patients in this study, and some other studies by our group (137, 136), presented a large variety of associated symptoms such as anxiety, dyspepsia, neurovegetative type of hyperglycemic curve and dermographism. All these symptoms and signs may be directly of indirectly related to target activity of catecholamines and/or other hormones. Since cyclic AMP was demonstrated to be a mediator for the activity of many of these hormones (221), it is conceivable that some more generalized abnormality in the cellular regulation, situated at the level of the adenyl cyclase - cyclic AMP system, may be common to all these patients.

Mendlowitz stated that ". . . the phenomenon of increased vascular responsiveness in essential hypertension must, in part at least, be considered to be of biochemical rather than structural origin". (182). We believe that results in this thesis, demonstrating some differences in cyclic AMP excretion in patients with labile hyperkinetic hypertension, present further evidence of biochemical disturbance in essential hypertension. The selection of patients with this particular type of hypertension was done purposely, as their clinical manifestations were compatible with the hypothesis involving an abnormality of the "second messenger" system. Any general implication for the role of this system in the pathogenesis of essential hypertension cannot however be made at the present time.

It will be the purpose of further studies, to which the author of this thesis feels dedicated, to elaborate upon the possible mechanism of defect in the transmission of hormonal information in hypertension.

VI - CLAIMS TO ORIGINALITY

A - Three methods based on different principles for the measurement of cyclic AMP were adopted and compared. They have been evaluated with respect to precision, simplicity and low cost. The protein binding assay was considered to be the best, secondly the radioimmunoassay and the enzymatic assay having the lowest grading.

B - The effect of posture and of isoproterenol infusion on cyclic AMP excretion was studied for the first time in control subjects and hypertensive patients, particularly those presenting labile hyperkinetic hypertension.

C - Both stimuli applied lowered cyclic AMP excretion in control subjects, while an increase was noted in patients. The correlations between the creatinine and cyclic AMP excretions were different in control subjects and patients in both positions; the infusion of isoproterenol led to a disappearance of the previously positive correlations.

D - The administration of propranolol rendered the postural reactivity of patients similar to that of control subjects with respect to the excretion of cyclic AMP and its correlation with urinary creatinine.

E - The presented results suggest the possibility of an abnormality localized at the level of β-adrenergic receptor - adenyl cyclase system in patients with labile hypertension.

VII - SUMMARY

Labile hyperkinetic hypertension is characterized by β-adrenergic hyperactivity accompanied by increased responsiveness of renin to upright posture. Beta-adrenergic receptors are related to the cyclic AMP generating enzyme, adenyl cyclase.

In order to evaluate a possible relationship between the cyclic AMP and the circulatory hyperactivity, urinary excretion of cyclic AMP was measured using three different methods in a total of 15 control subjects and 24 patients with labile hyperkinetic hypertension. Urines were collected during two four hour periods in recumbent and upright position and after β-adrenergic stimulation with isoproterenol. The excretion of cyclic AMP decreased in upright position with control subjects and increased in patients. The same divergent response was observed after isoproterenol infusion in both groups of subjects. The treatment by propranolol normalized the response in urinary cyclic AMP to upright posture in patients.

The different response to stimuli in patients with labile hyperkinetic hypertension may be secondary to 1) a disturbance in the mechanism regulating the excretion of cyclic AMP, or, in a more general sense, to 2) a disturbance in the reactivity of adenyl cyclase to hormonal stimulation, or to 3) a defect in the metabolic fate of cyclic AMP.

VIII - RESUME

L'hypertension labile hyperkinétique est caractérisée par une hyperactivité β-adrénergique, accompagnée d'une augmentation de la réaction posturale de la rénine. Les récepteurs β-adrénergiques font partie de l'adényl cyclase, l'enzyme génératrice de l'AMP cyclique.

Afin d'établir les relations possibles entre l'AMP cyclique et l'hyperactivité circulatoire, l'excrétion de l'AMP cyclique a été mesurée par trois méthodes différentes chez 15 sujets de contrôle et 24 patients avec hypertension labile hyperkinétique. Les urines ont été recueillies lors de deux périodes de quatre heures dans les positions couché et debout et après une stimulation β-adrénergique avec l'isoprotérénol. Tandis que l'excrétion de l'AMP cyclique a diminué debout chez les sujets de contrôle, elle a augmenté chez les patients. La même réaction opposée a été observée chez ces deux groupes lors de l'infusion d'isoprotérénol. Le traitement au propranolol a normalisé la réponse posturale de l'AMP cyclique chez les patients.

Les réactions anormales des patients aux deux stimuli étudiés peuvent être dues à 1) une perturbation du mécanisme excrétoire de l'AMP cyclique ou 2) à un défaut généralisé de l'activité de l'adényl cyclase en réponse à la stimulation hormonale ou 3) à un défaut dans la régulation du métabolisme de l'AMP cyclique.

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