SPECIAL ARTICLE

Some Aspects of the Biological Role of Adenosine 3',5'-monophosphate (Cyclic AMP)

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SUMMARY

Cyclic AMP (adenosine 3',5'-monophosphate or cyclic adenylylate) has now been established as a second messenger mediating many of the effects of a variety of hormones. Several of the metabolic effects mediated by cyclic AMP are discussed, and it is suggested that certain other ("functional" or "mechanical") hormonal effects may be similarly mediated. In particular, the evidence presented supports the hypothesis that the positive inotropic response to the catecholamines is mediated by cyclic AMP. Although knowledge of the biological role of cyclic AMP has not been widely applied clinically, sufficient knowledge has now accumulated to make research in this area desirable.

Additional Indexing Words:
Catecholamines  Steroidogenesis  Inotropism  Lipolysis  Glucagon
Methylxanthines  Myasthenia gravis  Adrenal disorders
Adrenergic β-receptors  Adenyl cyclase  Phosphorylase in liver
Phosphodiesterase

Cyclic AMP (adenosine 3',5'-monophosphate or cyclic adenylylate) was initially discovered as the intracellular mediator of the glycogenolytic effect of epinephrine and glucagon in the liver, but it has since come to be recognized as a second messenger mediating a variety of hormonal effects. The second messenger concept is illustrated in figure 1. According to this concept, the first messengers, the hormones themselves, travel from their cells of origin to the cells of their target tissues to cause therein an alteration in the intracellular level of a second messenger. It may be noted that in some cases, as in neuro-humoral transmission within the autonomic nervous system, and also in the case of pancreatic glucagon acting on the pancreatic beta cells, the first messenger may not have far to travel. (The classical definition of hormones as agents which are transported via the bloodstream seems unnecessarily restrictive from the biochemical point of view. A potentially more useful definition was proposed some years...
ago by Huxley, who suggested that hormones be regarded as agents which transfer information from one set of cells to another for the good of the cell population as a whole.

The formation of cyclic AMP from ATP (adenosinetriphosphate) is catalyzed by adenylyl cyclase, which in at least some cases is located in the cell membrane. Adenylyl cyclase is affected by different hormones in different tissues and is thus seen to function both as a discriminator for environmental signals and as a signal generator. By this mechanism, the initial extracellular signal, represented by the first messenger, is changed to a signal (the second messenger) which is capable of acting within the cell. This signal may undergo amplification in a variety of ways, the ultimate response depending on the enzymatic profile of the type of cell involved. The cybernetic aspects of hormone action have been discussed more extensively by Hechter and his associates. The only second messenger which has been identified to date is cyclic AMP, but it seems likely that other second messengers will eventually be discovered. The possibility that other cyclic nucleotides may function in this capacity will be discussed in a later section.

Before going on to some of the newer data, which may be of special interest to those engaged in cardiovascular research, a brief review of some of the early experiments which led to the discovery of cyclic AMP may be worthwhile.

**Early Experiments**

One of the most important components of the hyperglycemic response to epinephrine and glucagon is an increase in the rate of
release of glucose from the liver. After it was established that liver slices were suitable for the study of this effect, one of the first questions to be asked was whether glucose was pumped out of the liver cells when the hormones were added or did it accumulate and overflow because of an increased rate of production? The latter conclusion was suggested by experiments which showed that cells stimulated by epinephrine or glucagon contained much more glucose than the controls. That the breakdown of glycogen in response to these hormones was the result of phosphorolysis rather than hydrolysis was indicated by several lines of evidence, among them the finding that the glycogenolysis was associated with the formation of monosaccharides instead of the larger units which would have been expected had amylase activity been stimulated.

Figure 2 shows the results of some experiments involving the measurement of hexose monophosphate levels in liver slices. These experiments showed that the concentrations of both glucose-1-phosphate and glucose-6-phosphate increased when the hormones were added. Analysis of these and other data led to the conclusion that phosphorylase, rather than phosphoglucomutase or glucose-6-phosphatase, was rate-limiting in the conversion of glycogen to glucose, and that glucagon and epinephrine acted somehow to stimulate the activity of this enzyme. This conclusion was probably premature, in view of the later discovery of glycogen synthetase, but it nevertheless seems to have been substantially correct.

Further investigation of the hormonal effect on the phosphorylase system showed that the concentration of active enzyme in liver slices was increased by glucagon and epinephrine. This was demonstrated directly by measurements of phosphorylase activity in homogenates or extracts prepared from slices which had been incubated with and without the hormones. The level of phosphorylase in liver slices was shown to represent a balance between enzymatic inactivation and activation, and the change from the inactive to the active form in response to the hormones was found to be rapid. At this point reactivation could be obtained only in slices and not in broken cell preparations.

The inactivation process could be studied in such preparations, however, and liver phosphorylase and the enzyme inactivating it were prepared in purified form from dog liver. The product of the reaction, inactive phosphorylase, differed from muscle phosphorylase in that its activity was not greatly increased in the presence of 5'-AMP. The other product of the reaction was found to be inorganic phosphate, the formation of which paralleled the formation of inactive phosphorylase (2 moles of phosphate per mole of enzyme), with no apparent change in the molecular weight of the protein. These findings were just recently confirmed. These and other experiments led to the conclusion that the inactivating enzyme was a relatively specific phosphatase.

This conclusion in turn suggested that the activation of phosphorylase might consist of a phosphorylation of the inactive enzyme. Experiments were then designed to see if...
slices of liver would incorporate phosphate into the inactive enzyme during the process of activation. The results, some of which are illustrated in figure 3, showed clearly that phosphorylation did occur and that it was stimulated by epinephrine and glucagon. It later became possible to study this process in broken cell preparations, and a requirement for ATP and Mg++ was established. The enzyme which catalyzed the reaction was purified and named "dephosphophorylase kinase." This name never became very popular and was possibly a subconscious factor in a later decision to refer to adenosine 3',5'-monophosphate as cyclic AMP.

Subsequent events in this story have been reviewed relatively recently.1,19 In brief summary, then, an effect of epinephrine and glucagon in broken cell preparations was eventually obtained, but not if the homogenates were centrifuged and the precipitate removed. This is illustrated in figure 4. It was thus found that epinephrine and glucagon did not activate the phosphorylase in the soluble system, but instead reacted with the particulate material to make a heat-stable factor which was in turn responsible for the activation of phosphorylase.20 This factor was soon identified as cyclic AMP.21,22 The ability of cyclic AMP to stimulate the activation of liver phosphorylase constitutes the basis of our present assay system for this compound.23

The components known to be involved in the control of intracellular levels of cyclic AMP are illustrated in figure 5. Adenyl cyclase was found to be widely distributed,24 and indeed has been found in all nucleated mammalian cells which have been studied. Its location in the cell membrane6 has already been mentioned. In some tissues it may be located primarily in the sarcoplasmic reticulum, as appears to be the case in white skeletal muscle.25 Distribution studies with brain tissue are compatible with a synaptic localization,26 and extrapolation from studies with pineal gland homogenates27 suggests the likelihood of a postsynaptic site. Adenyl cyclase from most sources is sensitive to stimulation by fluoride,9,24 at least in broken cell preparations, and the other product of the reaction has been identified as pyrophosphate.28

Adenyl cyclase from mammalian sources has resisted most attempts to purify it. The enzyme from brain has been carried through lyophilization and lipid extraction with the retention of hormonal sensitivity, and has been solubilized to some extent.24 The possibility is recognized that it may not be possible to obtain mammalian adenyl cyclase in a soluble form with the retention of full hormonal sensitivity. It seems possible, in other words, that a certain conformational pattern, possible only within the highly organized framework of the intact cell membrane, is necessary for the enzyme to be activated by hormones. However, this possibility should by no means be regarded as a probability. Indeed, consideration of certain ideas which were regarded as highly improbable at one time, such as the idea that urea could be synthesized in a test tube or the more recent idea that enzymes might...
Effect of epinephrine (E) and glucagon (G) on phosphorylase activation in whole and fractionated cat liver homogenates. Homogenates or fractions thereof were incubated with ATP and Mg²⁺ in the presence and absence of epinephrine and glucagon. Inactive liver phosphorylase (dephospho-LP) was added when indicated. The supernatant fraction used in this experiment was the 1200 × g supernatant fraction. LP activity was assayed before and after 10 minutes' incubation at 30 C. The bars represent the amount of LP formed during the incubation period; the crosshatched areas represent the increased LP formation above that of the control. From Rall and associates.20

Table 1

Some Hormonal Responses Involving Cyclic AMP

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Response</th>
<th>Criteria*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catecholamines</td>
<td>Phosphorylase activation (liver)</td>
<td>+</td>
<td>1, 3, 37</td>
</tr>
<tr>
<td></td>
<td>Phosphorylase activation (heart)</td>
<td>+</td>
<td>37, 38</td>
</tr>
<tr>
<td></td>
<td>Positive inotropic response</td>
<td>+</td>
<td>3, 5, 151</td>
</tr>
<tr>
<td></td>
<td>Lipolysis</td>
<td>+</td>
<td>39, 40</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Phosphorylase activation (liver)</td>
<td>+</td>
<td>1, 3</td>
</tr>
<tr>
<td></td>
<td>Insulin release</td>
<td></td>
<td>6, 41</td>
</tr>
<tr>
<td>ACTH</td>
<td>Steroidogenesis (adrenal cortex)</td>
<td>+</td>
<td>42, 43</td>
</tr>
<tr>
<td>LH (ICSH)</td>
<td>Steroidogenesis (corpus luteum)</td>
<td>+</td>
<td>44, 45</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>Steroidogenesis (zona glomerulosa)</td>
<td>+</td>
<td>46</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Permeability changes</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>TSH†</td>
<td>Thyroid hormone production</td>
<td>+</td>
<td>47, 48, 49</td>
</tr>
<tr>
<td>MSH†</td>
<td>Melanocyte dispersion</td>
<td>+</td>
<td>50</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Phosphofructokinase activation</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>Gastrin</td>
<td>HCl production</td>
<td></td>
<td>52, 53</td>
</tr>
</tbody>
</table>

*Criterion 1 = broken cell preparations; 2 = intact tissue; 3 = potentiation by methylxanthines; and 4 = production of response by cyclic AMP in a and b; a = intact tissue; b = broken cell preparation. A negative sign does not necessarily imply a negative result but only that the criterion has not been established.

†TSH = thyroid-stimulating hormone; MSH = melanocyte-stimulating hormone.
be proteins, suggests that the prediction of the impossible is a difficult business. An interesting development in this regard has been the isolation of a soluble adenyl cyclase from Brevibacterium liquefaciens.\textsuperscript{29} This enzyme is stimulated by pyruvate and certain related \( \alpha \)-keto acids but was not affected by any of the mammalian hormones which were tested.\textsuperscript{29} The biological significance of this has yet to be established.

The phosphodiesterase is more widely distributed throughout the cell than is adenyl cyclase, being located partly in the particulate material and partly in the soluble fraction.\textsuperscript{26, 30, 31} Several drugs, including the methylxanthines,\textsuperscript{30} certain benzothiadiazine derivatives,\textsuperscript{32, 33} and puromycin,\textsuperscript{34} are capable of inhibiting the phosphodiesterase, and it is possible that some of the pharmacological effects of these drugs are related to this action. Among cellular constituents, the enzyme is subjected to inhibition by ATP, pyrophosphate, and citrate.\textsuperscript{31} Imidazole stimulates the enzyme in vitro,\textsuperscript{30} and it is possible that this effect occurs in vivo as well.\textsuperscript{35} The enzyme which is effective against cyclic AMP is relatively specific for purine derivatives, but an enzyme has been obtained from dog heart which has a relatively high specificity for cyclic uridylic acid (cyclic UMP).\textsuperscript{36} This enzyme is more sensitive to inhibition by the methylxanthines and less sensitive to stimulation by imidazole than is the phosphodiesterase which preferentially attacks cyclic AMP.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5.png}
\caption{Figure 5}
\end{figure}

\textbf{Biological Role of Cyclic AMP}

A variety of hormones are now known to exert at least some of their effects by causing an alteration in the intracellular level of cyclic AMP. Some of the hormones for which there is evidence of this are listed in table 1. In all of these cases the hormones seem to act by stimulating adenyl cyclase, which, as mentioned, is responsive to different hormones in different tissues. To establish this as the mechanism by which a given response is produced, it is desirable to have several criteria satisfied.

First, adenyl cyclase in broken cell preparations should respond to the same hormones which are effective in the intact tissue. If analogues of the hormones are available, then the order of potency should be the same in vitro as it is in vivo. If competitive antagonists are available, they also should behave similarly. Of course it is recognized that qualitative differences may occur, in view of the many differences which may exist between broken cells and intact tissue (such as in the rates of hormone metabolism, tissue uptake, and the like). In fact, however, the agreement in most instances has been remarkably good. If significant exceptions do occur, then these must be explained. A point to be considered here is that some tissues may contain several types of cells in which the adenyl cyclases differ with respect to the hormones which stimulate them.

Secondly, the level of cyclic AMP in intact tissues should change appropriately in response to hormonal stimulation. This criterion should preferably be satisfied under conditions in which the physiological response can be simultaneously monitored. The change in the level of cyclic AMP should precede or at least not follow the physiological response. Cyclic AMP levels in intact tissues are subject to very rapid fluctuations, and the importance of rapid fixation in this regard cannot be overemphasized.

A third criterion which may be useful is that hormones which stimulate adenyl cyclase should be potentiated by drugs which inhibit phosphodiesterase activity. This criterion is obviously subject to several qualifications. In the case of broken cell preparations, it is clear that an active phos-
phosphodiesterase must be present. In all cases it is necessary to ascertain that the effects of phosphodiesterase inhibition are not being obscured by some other effect of the drug. Dose levels are also critical in attempting to satisfy this criterion. If the concentration of the hormone is such that the level of cyclic AMP is already high enough to produce a maximal response, then inhibiting the phosphodiesterase will not cause a further increase in the response.

Finally, it may be possible in some cases to mimic the effect of the hormone by the addition of exogenous cyclic AMP. This may not be possible in all cases because of the permeability problem, but if enough is known about the steps between the change in cyclic AMP and the final overall response, this criterion can often be satisfied by the use of broken cell preparations. In other cases one of the derivatives of cyclic AMP may be effective even though cyclic AMP itself is not. The reasons for this are not known. It is known that the derivatives are more resistant than cyclic AMP to the action of phosphodiesterase, and it is possible that they penetrate cell membranes more readily, at least in some tissues. The ability of the derivatives to produce a response will depend in part on the ability of the tissue to remove the substituent moieties. This accounts for the fact that in partially purified liver extracts, for example, the derivatives are less active than cyclic AMP in stimulating phosphorylase activation, whereas in liver slices they may be more active.

On the basis of these criteria, it is clear that many of the effects of the hormones listed in table 1 are mediated by cyclic AMP. Where all four criteria have been satisfied, then it can be said that this is well established. For each of these hormones we have selected one or more effects (listed in the second column) and for each of these we have indicated, in the next column, the number of criteria which have been satisfied. In the case of the fourth criterion, involving the production of an effect by cyclic AMP, we have distinguished whether this criterion was satisfied in intact tissue or by the use of a broken cell preparation. In those cases in which the cited references refer to less than the number of criteria shown in the third column, it can be understood that the other criteria have been established by unpublished data, either from our own laboratories or elsewhere. It should be noted that the list of effects in this table does not by any means include all of the effects in which cyclic AMP is probably involved. Some of the other effects of cyclic AMP have been discussed elsewhere, and a monograph covering all aspects of this subject is in preparation. It should also be noted that several other hormones, including insulin and the prostaglandins, may produce at least some of their effects by causing an alteration in the intracellular level of cyclic AMP. However, there is at the moment no clear evidence to indicate whether this results from a change in the activity of adenyl cyclase or of the phosphodiesterase.

Another point which could be made in this section, at the risk of stating the obvious, is that the identification of cyclic AMP as the second messenger in a given hormonal response does not mean that the response is well understood. In no case, for example, is it clear how the hormones act at the molecular level to stimulate adenyl cyclase. This has led to a certain amount of speculation concerning the possible relation of the receptor (that pattern of forces in or on the cell with which the hormone actually interacts) to adenyl cyclase. We have tended to favor the hypothesis that the receptor is an integral part of the adenyl cyclase system and have suggested that the adrenergic receptor may be an example of such a receptor. Other investigators have tended to favor the view that the receptor must be a separate entity, and that the adenyl cyclase system may be only one of many systems which are affected by it. In defense of our own position, it may be noted that in those hormonal responses which are mediated by cyclic AMP, the stimulation of adenyl cyclase is the earliest event known to occur.
and attempts to measure an earlier event have been uniformly unsuccessful. The retention of hormonal sensitivity by adenyl cyclase through several stages of purification has been interpreted by us as support for the unitary hypothesis, whereas the eventual loss of hormonal sensitivity has been interpreted by others as support for the alternate view. It may be useful in this regard to consider briefly the studies which have been carried out with aspartate transcarbamylase. The enzyme from E. coli has been extensively purified and its molecular weight determined. It has been found that under appropriate conditions the enzyme can be dissociated into two types of subunits. One subunit possesses all of the catalytic activity of the undenatured enzyme but is not subject to inhibition by CTP, which in E. coli is the normal allosteric inhibitor of the enzyme. The other subunit has no catalytic activity but possesses all of the binding sites for CTP. These elegant studies were carried out by Gerhart and Schachman. In certain other species, however, it has not yet been possible to purify aspartate transcarbamylase very extensively and still retain the enzyme's sensitivity to allosteric inhibition. The parallel between these studies and our own experience with adenyl cyclase has seemed to us striking, or at least suggestive. These and other considerations, including the cellular location of adenyl cyclase, have in any event led us to regard our own view as a reasonable one, although it is obviously far from established.

On the other side of the membrane there is the problem of how cyclic AMP acts to produce the effects which it is known to be capable of producing. In the several cases which have been studied in detail, such as phosphorylase activation, ATP is a component of the reaction which is affected. In certain other cases, such as the permeability changes which are seen in the toad bladder, virtually nothing is known about the basic nature of the process involved. It is perhaps not too much to hope, however, that the identification of cyclic AMP as the mediator of these responses may lead to a clearer understanding of the responses themselves.

At this point we might briefly consider two of the responses which have just recently been placed in the "well-established" category. These are the lipolytic response of adipose tissue to a variety of hormones and the steroidogenic response of the adrenal cortex to ACTH. Following this we will turn to the cardiovascular system, with emphasis on the positive inotropic response to the catecholamines. Finally we would like to make a few general remarks, including a brief discussion of the possible clinical implications of this research.

The Lipolytic Response

Lipolysis (the breakdown of triglycerides to free fatty acids and glycerol) has been studied in detail in the rat epididymal fat pad and has been found to be stimulated by the catecholamines, ACTH, glucagon, TSH, and several other hormones. The lipolytic actions of these hormones can be antagonized by a variety of agents, including insulin, the prostaglandins, nicotinic acid, and adrenergic blocking agents.

The lipolytic response was of great interest from the standpoint of the biological role of cyclic AMP, for all of the hormones with lipolytic activity were known or suspected to have actions mediated by cyclic AMP in the tissues with which they were more commonly associated (table 1). In addition, there seemed to be little possibility that phosphorylase activation was involved in the breakdown of triglycerides. We had felt for some time that the importance of phosphorylase activation in hormonal effects in general had been overemphasized.

All four criteria have been applied to the lipolytic response of adipose tissue and have been met. An epinephrine-sensitive adenyl cyclase system was identified in broken cell preparations of rat epididymal fat pads. Adenyl cyclase activity in preparations of lysed isolated fat cells was stimulated by ACTH and glucagon as well as by epineph-
rine, and the effect of epinephrine was antagonized by pronethanol.

Perhaps the most extensively studied criterion has been the second, that is, the relationship between intracellular cyclic AMP levels and lipolysis. Both lipolysis and the concentration of cyclic AMP were increased in fat pads incubated with epinephrine, and the degree to which lipolysis was stimulated was directly related to the level of cyclic AMP in the cell (except at very high levels, where another component of the lipolytic mechanism was saturated and cyclic AMP was no longer rate-limiting). In addition, caffeine, which had been shown by Vaughan and Steinberg to act synergistically with epinephrine on lipolysis, thus satisfying our third criterion, was found to act in the same fashion on cyclic AMP formation. The effect of epinephrine on cyclic AMP levels preceded any detectable increase in the rate of release of free fatty acids (FFA), and the effects of β-adrenergic blocking agents on lipolysis were reflected in their effects on cyclic AMP levels. The α-adrenergic blocking agents, which in high concentrations may act as noncompetitive antagonists of a variety of lipolytic hormones, probably act by interfering with the action rather than the formation of cyclic AMP. Phentolamine, for example, does not prevent the accumulation of cyclic AMP in the epididymal fat pad in response to catecholamines, but Aulich and associates have shown that it does antagonize the lipolytic action of the dibutyryl derivative of cyclic AMP. Interference with the action of cyclic AMP is probably not the mechanism of α-adrenergic blockade in general, but several anomalous actions of the α-adrenergic blocking agents may be understandable on this basis. These would include effects of dihydroergotamine in the rat liver and, possibly, in the rat uterus as well. This does not exclude the possibility that in some tissues these drugs may interfere with the formation of cyclic AMP, either in addition to or instead of an action at some later point. Such an effect of ergotamine, for example, was seen in dog liver homogenates.

Insulin, which had been shown by Jungas and Ball to antagonize the effects of the catecholamines on lipolysis, caused rapid and dramatic lowering of cyclic AMP levels in fat pads and isolated fat cells which had been stimulated by lipolytic hormones. The anti-lipolytic and cyclic AMP lowering effects of insulin have now been studied in detail, and were found to be well correlated (J. D. Corbin, unpublished observations).

The prostaglandins have also been found to have anti-lipolytic activity which is mediated by decreased intracellular cyclic AMP levels. In isolated fat cells prostaglandin E1 (PGE1) antagonized the actions of the catecholamines, ACTH, glucagon, and TSH. In addition, the potencies of the different prostaglandins on cyclic AMP formation are closely related to their known antilipolytic potencies. These agents are effective at very low concentrations. For example, PGE1 caused a 50% inhibition of 5.5 μM epinephrine at a concentration of 0.004 μM.

Insulin and the prostaglandins lower cyclic AMP levels by mechanisms which are as yet unknown. Jungas found that insulin lowered cyclic AMP accumulation in homogenates of fat pads which were exposed to insulin before rupture, but that it was ineffective when added after homogenization. We also have been unable to elicit effects in broken cell preparations, not only with insulin but also with the prostaglandins. We can therefore only speculate about the mechanisms, which might include an inhibition of the cyclase, stimulation of the phosphodiesterase, or perhaps an indirect mechanism involving the production of another second messenger, which could then produce one or both of the effects mentioned. Another possibility is that the effects on cyclic AMP are secondary to some change in the structure of the cell membrane.

Finally, the ability of exogenous cyclic AMP to stimulate the release of FFA and glycerol by fat pads and isolated fat cells has been demonstrated and confirmed. In general, cyclic AMP itself has been ineffective or only slightly stimulatory, but the
Summary of some interrelationships involving cyclic AMP in adipose tissue.

The Steroidogenic Response

The steroidogenic action of ACTH on the adrenal cortex is one of the more intensively studied hormone actions in which cyclic AMP plays a role. All four criteria have been met, and, while the mechanism by which cyclic AMP translates and amplifies the signal carried by ACTH is not entirely clear, it now seems well established that cyclic AMP does the job.

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As shown schematically in figure 7, ACTH acts specifically with the adenyl cyclase system. Cell-free preparations of adenyl cyclase from rat adrenals and beef adrenal cortex responded to the addition of ACTH with increased cyclic AMP accumulation, while epinephrine and glucagon were without effect. These data satisfy (in part at least) our first criterion.

The second criterion has also been established. Haynes and associates found some years ago that the addition of ACTH to beef adrenal slices caused increased cyclic AMP levels (and phosphorylase activation) as well as increased rates of corticoid production. More recently, Grahame-Smith and associates have further studied the relationship between cyclic AMP and steroidogenesis in the rat adrenal in vitro and in vivo. Within limits, a direct proportionality was found between the level to which cyclic AMP was increased by ACTH and the amount of steroid hormone produced. The qualitative specificity was also examined in detail, using analogues of ACTH, and the potencies of these analogues in producing increased levels of cyclic AMP were found to be the same as their potencies as steroidogenic agents. Temporally, it was found that cyclic AMP levels were increased in response to ACTH before any increase in the rate of steroidogenesis could be detected.

The finding that ACTH is capable of increasing the concentration of cyclic AMP in the adrenal cortex to a level higher than necessary for a maximal response is similar to the findings with epinephrine in adipose tissue and also with glucagon in the liver. The physiological significance of this "extra" cyclic AMP seems dubious, for it is unlikely that the plasma levels of the hormones involved ever reach the levels necessary for the production of such high concentrations of cyclic AMP. The phenomenon is nevertheless of some theoretical interest and might even be taken as a good illustration of the pharmacological concept of spare receptors.

Bieck and Westermann have reported that theophylline does not increase the steroidogenic effect of ACTH in the rat. Halkerston and associates studied this in vitro and found that, although higher concentrations of theophylline were inhibitory, a concentration of $10^{-2}$M caused a small but statistically significant potentiation of ACTH. Further experiments showed that the inhibitory effect of theophylline was associated with an inhibition of protein synthesis, which is especially important in this case because of the evidence that protein synthesis is involved in the steroidogenic effect of cyclic AMP. The existence of a "rapidly turning-over protein" has been postulated to account for the inhibitory effects of other inhibitors of protein synthesis.

The fourth criterion was initially satisfied in beef adrenal slices by Haynes and associates. The steroidogenic effect of exogenous cyclic AMP has since been demonstrated by a number of investigators in several different species both in vitro and in vivo. These data are reviewed in detail elsewhere.

The mechanism by which cyclic AMP acts to stimulate steroidogenesis is unclear at the present time. An important rate-limiting step appears to be the conversion of cholesterol to pregnenolone, and it is this step which seems to be ultimately stimulated by cyclic AMP. The possible participation of a "rapidly turning-over protein" has been mentioned, but the nature of its participation, or the mechanism by which its synthesis might be affected by cyclic AMP is unknown. The recent report by Khairallah and Pitot that cyclic AMP is capable of stimulating the release of newly synthesized protein from liver polysomes, may be of interest in this regard. Haynes' earlier hypothesis that phosphorylase activation might be important, leading as it would to increased levels of NADPH (which is required for steroid hydroxylations) has still not been ruled out. Even if phosphorylase activation is not involved in the initiation of the steroidogenic response, it certainly might play an important supportive role.

Other steroidogenic hormones probably act by a fundamentally similar mechanism. The
action of LH on the corpus luteum has been studied in detail, and there is now evidence that the action of this hormone on the interstitial cells of the testes is also mediated by cyclic AMP.

**Possible Role in the Cardiovascular System**

The identification of cyclic AMP as the second messenger mediating several of the metabolic effects of the catecholamines suggested that some of the other effects of these agents might also be mediated by cyclic AMP. The classification of the effects of the catecholamines into those which are metabolic and those which are something else ("functional" or "mechanical" effects) is of course recognized as highly artificial. Metabolic effects can be measured chemically, usually with the aid of an instrument designed for this purpose, the final observation often being of the position of a pointer on a dial. The other effects differ in that the chemical events responsible for them are not well understood, and consequently are measured by changes in the physical properties of the tissues themselves.

It is possible that cyclic AMP plays a much more important role in the regulation of cardiovascular function than is presently realized. Now a considerable amount of evidence indicates that in those tissues where adrenergic β-receptors occur, these receptors are closely associated with (if not an integral component of) the adenylyl cyclase system. With reference to the cardiovascular system, the corollary of this would be that the positive inotropic, positive chronotropic, and vasodepressor effects of sympathetic stimulation may be mediated by increases in the level of cyclic AMP, in the cells responsible for these effects. In addition, there is now some evidence to support the hypothesis that the effects of α-receptor activation may be related to a decrease in the intracellular level of cyclic AMP. This was suggested by Turtle and associates on the basis of their experiments on the effects of theophylline and adrenergic blocking agents on insulin release. Porte had previously shown that the stimulation of insulin release in response to catecholamines was mediated by β-receptors, while suppression of insulin release was mediated by α-receptors. Other tissues in which the effects of α-receptor activation are the opposite to those of cyclic AMP include the toad bladder and frog skin. The implication of this for the cardiovascular system is that sympathetic vasoconstriction could be mediated by a decrease in the level of cyclic AMP in vascular smooth muscle.

Vascular smooth muscle has not been extensively studied from the point of view of cyclic AMP levels. It will not be further discussed, therefore, except to mention that Bartelstone and associates and others have suggested that sympathetic vasoconstriction might be mediated by an increase, rather than a decrease, in the intracellular level of cyclic AMP. This had also been considered as a possibility by us. However, the data on which this suggestion was based was highly equivocal, and, as mentioned, most of the evidence now favors the view that α-receptor effects are mediated by a decrease in the level of cyclic AMP. This is also in line with the effects of the methylxanthines, which antagonize the smooth muscle contracting effects of the catecholamines. Bartelstone and associates reported several instances in which small concentrations of theophylline, under certain special conditions, actually seemed to increase the contraction of smooth muscle in response to catecholamines. This could mean that in smooth muscle, as in so many other tissues, the methylxanthines may have more than one action. These speculations concerning the α-receptor could be extended to several other vasoconstrictor hormones which have been shown to influence cyclic AMP levels in other tissues. There is at present no experimental evidence that any of these hormones are capable of decreasing the level of cyclic AMP in vascular smooth muscle, but we believe that this possibility may be worth considering in future investigations.

The positive inotropic response to the catecholamines will be discussed in more detail in the next section. The positive chronotropic response also may be mediated by...
cyclic AMP, but this response has been little investigated biochemically, partly because of the small amount of tissue involved in it relative to the total mass of the heart. It may be worth mentioning, however, that Blinks and Kaumann, using isolated cardiac muscle, have found that the dose-response curves for inotropic and chronotropic effects are very similar, and are affected in almost identical fashion by \( \beta \)-adrenergic blocking agents. This might suggest that not only are the receptors themselves similar but that the relationship between the stimulus (cyclic AMP, if we are correct) and the response is also very similar. This would not necessarily have been predicted on the basis of the adenyl cyclase hypothesis but is perhaps understandable when considered from an evolutionary point of view. This interpretation is complicated in mammals by the numerous reflex effects which occur in response to the direct cardiovascular effects of the catecholamines. It is possible that in the distant past, however, survival was favored as the inotropic and chronotropic responses evolved in unison.

Levine and Vogel reported a positive chronotropic effect, in dogs as well as in humans, in response to the injection of cyclic AMP. Isolated cardiac muscle has proved to be one of the least permeable to cyclic AMP of all tissues studied, and, assuming that this lack of permeability extends to the pacemaker cells as well, it seems possible that the response noted by Levine and Vogel may not have been a direct effect of cyclic AMP. James studied the effect of cyclic AMP in dogs by direct perfusion of the sinus node. Although cyclic AMP was considerably less potent than the other adenosine derivatives studied, including ATP and 5'-AMP, it was qualitatively similar in that it produced a negative chronotropic effect. This observation raises the possibility that even if enough cyclic AMP were to penetrate the myocardial cells to produce an effect, this might be obscured by other effects occurring at the cell membrane. In our own studies with the isolated perfused working rat heart, we were unable to produce a consistent effect with either cyclic AMP or several of its derivatives, and the results with isolated hearts from other species have apparently also been inconsistent.

**Positive Inotropism**

The first evidence that cyclic AMP might be involved in the positive inotropic response to the catecholamines came from studies on phosphorylase activation in the perfused heart. In these studies, which have been reviewed by Haugaard and Hess, a good correlation was obtained between the inotropic response and the activation of phosphorylase when the effects of different sympathomimetic amines and adrenergic blocking agents were compared, suggesting that both responses were mediated by adrenergic \( \beta \)-receptors. It was later shown, however, that the two responses could be dissociated to at least some extent. Small doses of epinephrine were found to be capable of causing substantial increases in contractile force with little or no change in phosphorylase activity.

**Figure 8**

The relation of catecholamine concentration to the formation of cyclic AMP by dog heart adenyl cyclase. Hypotonic myocardial suspensions were incubated with and without catecholamines for 12 minutes at 30 C. The increase in the formation of cyclic AMP in the presence of various concentrations of epinephrine, norepinephrine, or isoproterenol is plotted against the concentration of catecholamine. There was 7.7 \( \times \) 10\(^{-5} \) \( \mu \) mole/ml of cyclic AMP formed with no catecholamine added. From Murad and associates.
Also, when time courses were studied, the inotropic response was found to precede the change in phosphorylase activity. The data suggested that although the two responses might have a common mediator, or second messenger, the activation of phosphorylase was probably not responsible for the increase in contractile force. The opposite possibility, that the increased force might somehow lead to phosphorylase activation, has also been ruled out. (Increased cardiac work is associated with an increase in the rate of glycolysis, but this seems to be caused primarily by the allosteric activation of phosphorylase $b$ and other enzymes, rather than by the conversion of phosphorylase $b$ to phosphorylase $a$. The same appears to be true in large measure of the glycogenolytic effect of anoxia, although under certain conditions the production of anoxia may be associated with the release of catecholamines.

More direct evidence that cyclic AMP was involved in the cardiac effects of the catecholamines came from studies on adenylyl cyclase in broken cell preparations from dog heart. As illustrated in figure 8, the relative potencies of a series of catecholamines in stimulating adenylyl cyclase were found to be similar to their relative potencies as inotropic agents in vivo. Isoproterenol may be even more potent in vivo than is suggested by the in vitro experiments, since it is less readily taken up by neuronal tissue than are the naturally occurring catecholamines. These effects were blocked competitively by DCI and pronethalol. Isopropylmethoxamine (IMA), a $\beta$-adrenergic blocking agent which is less potent than pronethalol in preventing the inotropic response to isoproterenol was also less effective than pronethalol in preventing the stimulation of adenylyl cyclase in vitro. Thus the first of our four criteria was established.

Tissue levels of cyclic AMP have been measured in isolated perfused working rat hearts, in isolated rat and rabbit hearts perfused by the Langendorff technique, and in the rat heart in situ and were found to be elevated in all cases following the administration of epinephrine. The studies in the rat heart, illustrated in figure 9, revealed that the activation of adenylyl cyclase was an extremely rapid process, preceding both the inotropic response and the activation of phosphorylase. The increase in cyclic AMP may occur even more rapidly than is indicated by the Langendorff technique, and in the rat heart in situ and were found to be elevated in all cases following the administration of epinephrine. The studies in the rat heart, illustrated in figure 9, revealed that the activation of adenylyl cyclase was an extremely rapid process, preceding both the inotropic response and the activation of phosphorylase. The increase in cyclic AMP may occur even more rapidly than is indicated.

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*CIC = dichloroisopropyl-arterenol (1-[3,4-dichlorophenyl]-2-isopropylaminoethanol hydrochloride).

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**Figure 9**

Effect of a single dose of epinephrine in the isolated perfused working rat heart. Hearts were frozen 3, 15, 30, 45, 60, and 120 seconds after injection of epinephrine. Contractile force shown is average response of the hearts frozen at 60 and 120 seconds. Lower curve in phosphorylase panel shows lack of response to injected saline. From Robison and associates.
by these data, since Drummond and associates\textsuperscript{117} noted a ninefold increase within 1 second of the administration of epinephrine. Namm and Mayer,\textsuperscript{118} using a biopsy sampling technique, were unable to detect a significance change in cyclic AMP in response to epinephrine in the dog heart in situ. This was found to be due to the delay in freezing the sample. These studies by Namm and Mayer offer an impressive demonstration of the need for rapid fixation in experiments involving the measurement of intracellular cyclic nucleotide levels.

In our own experiments with the isolated perfused working rat heart, the administration of pronethalol was found to prevent the effects of epinephrine on both contractility and cyclic AMP levels.\textsuperscript{104} This effect of pronethalol has more recently been demonstrated in the rat heart in situ,\textsuperscript{118} and Drummond and associates\textsuperscript{117} showed that the activation of phosphorylase \textit{b} kinase, which is closely related to the level of cyclic AMP, was also blocked by pronethalol. By contrast, IMA, at the highest concentrations which we could use in the working rat heart without producing severe cardiotoxicity, prevented neither the rise in cyclic AMP nor the positive inotropic effect in response to a relatively large dose of epinephrine.\textsuperscript{104} This was an important experiment because Burns and associates\textsuperscript{119} had reported that a similar concentration of IMA in dogs prevented most of the metabolic responses to isoproterenol but did not affect the inotropic response. Since the metabolic responses were known to be mediated by cyclic AMP, it might have been predicted that in the rat heart IMA would prevent the increase in cyclic AMP in response to epinephrine but not the positive inotropic effect, thereby invalidating the hypothesis that this response is mediated by cyclic AMP. As summarized above, however, this was found not to be the case.

Further stimulated by the findings of Burns and associates,\textsuperscript{119} we measured the ability of IMA to prevent the stimulation of adenyl cyclase by isoproterenol in washed particulate preparations from dog heart and liver.\textsuperscript{8} Pronethalol and IMA were found to be roughly equipotent in the liver, but IMA was at least 10 times less effective in the heart. This is in line with the physiological data,\textsuperscript{115,119} and also with the concept that the \( \beta \)-receptor is closely associated with (if not an integral component of) the adenyl cyclase system.

Other investigators, using a variety of non-working cardiac muscle preparations,\textsuperscript{100,115,120} have shown that IMA is a weak \( \beta \)-adrenergic blocking agent and that the cardiac effects of small doses of the catecholamines can be completely prevented by relatively large doses of IMA. Kukovetz and Pöchl\textsuperscript{120} studied this in the isolated perfused guinea pig heart and found that the effects of the catecholamines on phosphorylase \( a \) levels and contractility were affected similarly by IMA. These and other studies by Kukovetz and Pöchl\textsuperscript{121} suggest that in the guinea pig heart the activation of phosphorylase and the enhanced contractile force may be more closely geared than in the rat or dog heart.\textsuperscript{105,106} Even in this species, however, it was possible to dissociate the two responses by the use of a low concentration of norepinephrine.\textsuperscript{121} Moran\textsuperscript{60} has reported that he and Mayer noted a transient inhibition by IMA of phosphorylase activation in the dog heart in situ in response to norepinephrine, but not in response to isoproterenol. The positive inotropic response was unaffected in either case. Kukovetz and Pöchl\textsuperscript{121} were unable to dissociate the inotropic response from phosphorylase activation in the guinea pig heart by the use of isoproterenol, even though, as mentioned, they were able to do so by the use of small doses of norepinephrine. They then made the interesting discovery that the cardiac effects of isoproterenol occurred more rapidly than those of norepinephrine, presumably a reflection of the greater potency of isoproterenol in stimulating cardiac adenyl cyclase.\textsuperscript{78}

The reasons for the lower potency of IMA in the heart are not known. A widely held concept in the past has been that all \( \beta \)-receptors (as defined by order of potency of agonists and competitive blockade by \( \beta \)-adrenergic blocking agents)\textsuperscript{122} are identical, although it is now recognized that this is probably not
true.\cite{5, 123, 124} As indicated above, the methoxamine derivatives have been of special interest in this regard, since they seem to have a high affinity for some \( \beta \)-receptors but low affinity for others. IMA, for example, effectively blocks most of the metabolic responses to the catecholamines in intact dogs\cite{119} and also blocks \( \beta \)-receptors in the uterus\cite{125} and bronchial smooth muscle,\cite{69} but is relatively ineffective in preventing the cardiovascular responses to the catecholamines.\cite{119, 125} Butoxamine\cite{126} and the dimethyl derivative of IMA\cite{127} have similar spectra of activity but are in addition capable of blocking \( \beta \)-receptors in vascular smooth muscle in doses which do not prevent the cardiac responses to the catecholamines.

Drummond and associates\cite{117} have studied phosphorylase \( b \) kinase activation in the perfused rat heart in response to catecholamines, in addition to measuring cyclic AMP levels. In contrast to their earlier studies with phosphorylase,\cite{106} they found that the activation of phosphorylase \( b \) kinase could not be dissociated from the inotropic response and that phosphorylase \( b \) kinase activity was in fact closely related to the level of cyclic AMP. Similar data were obtained by Namm and Mayer\cite{118} in the rat heart in situ. These investigators also found that in the dog heart in situ epinephrine could increase phosphorylase kinase activity at a dose which did not increase the level of phosphorylase \( a \). These data appear to indicate that in the cell the activity of phosphorylase \( b \) kinase must exceed a certain threshold before it can catalyze the conversion of phosphorylase \( b \) to \( a \). Other explanations are possible, however, and a final resolution of the problem must await further research.

The data in figure 9 illustrate the effect of allowing a single dose of epinephrine to be rapidly pumped through the heart. When the epinephrine is allowed to recirculate, the level of cyclic AMP still rises and falls, but it falls to an intermediate level which is dose-dependent.\cite{5} This is illustrated in the lower panel of figure 10. In these experiments the hearts were frozen 5 minutes after the addition of the indicated concentration of epinephrine. By this time the force of contraction had begun to decline due to the increase in rate.\cite{128} Peak contractile force could be maintained by crushing the right atrium and stimulating the heart electrically, but, since this procedure interfered with the rapid freezing of the heart, we chose instead to measure the inotropic effect at its peak, at whatever time this occurred, and these values are shown in the upper panel of figure 10. The important point to be made here is that the increase in the level of cyclic AMP could not be dissociated from the drug-induced increase in contractile force. The smallest concentrations of epinephrine with which we could obtain a measurable inotropic effect

![Figure 10](http://circ.ahajournals.org/)

**Figure 10**

*Effect of epinephrine on contractile force and cyclic AMP concentration in isolated perfused working rat hearts. Hearts were frozen 5 minutes after addition of indicated concentration of epinephrine, and cyclic AMP was measured as described previously.\cite{23} Points plotted in the upper panel represent peak systolic pressure developed following addition of epinephrine (as percentage of control pressure before addition of epinephrine). From Robison, Butcher, and Sutherland.\cite{5}*

*From Robison, Butcher, and Sutherland.*
also caused a significant increase in the intracellular concentration of cyclic AMP. These studies, together with the other studies mentioned in the foregoing paragraphs, have combined to help satisfy our second criterion.

Other cardiac effects produced by the catecholamines, in addition to those already mentioned, include a decrease in glycogen synthetase activity\textsuperscript{129, 130} and an increase in myocardial oxygen consumption. The effect on glycogen synthetase seems to be mediated by cyclic AMP,\textsuperscript{131} but this effect may be obscured in the beating heart by the opposite effect of decreasing glycogen levels\textsuperscript{130, 131} and possibly other factors.\textsuperscript{129} Under normal conditions the effect on oxygen consumption may be largely secondary to the increased work of the heart,\textsuperscript{132} but Challoner and Steinberg\textsuperscript{133} found that a significant increase in O\textsubscript{2} consumption (qO\textsubscript{2}) could be produced by epinephrine even in nonbeating hearts. On the basis of several lines of evidence, including the demonstration that intracellular FFA levels were increased in these hearts, Challoner and Steinberg suggested that the stimulation of qO\textsubscript{2} might be secondary to an increased lipolytic activity. This is supported by the recent finding that lipolytic activity in rat heart homogenates can be stimulated by the addition of cyclic AMP.\textsuperscript{134} It had been shown previously that cyclic AMP levels increase in response to epinephrine whether the heart is beating or not.\textsuperscript{5} The mechanism of lipase activation is not well understood, even in adipose tissue,\textsuperscript{79} but it would now appear that this effect of cyclic AMP may be common to a variety of tissues.\textsuperscript{134–136} The requirement for ATP and Mg\textsuperscript{++} suggests that the basic process may be similar to the activation of phosphorylase.\textsuperscript{83}

The cardiac effects of large concentrations of glucagon seem to be the same as those of epinephrine,\textsuperscript{136, 137} including the production of a positive inotropic effect.\textsuperscript{138} It has recently been shown in the perfused rat heart that these concentrations of glucagon increase the intracellular level of cyclic AMP.\textsuperscript{139} This effect of glucagon is not likely to be of physiological significance, because of the high concentrations required, but it is at least of some theoretical interest. Possibly the inotropic effects of several other hormones which are known to affect cyclic AMP levels in other tissues, such as the effects of histamine\textsuperscript{140} and the prostaglandins,\textsuperscript{141} may be produced by a similar mechanism. However, there is no direct evidence at the present time that these agents alter the level of cyclic AMP in the heart.

Potentiation by theophylline, our third criterion, was demonstrated for the positive inotropic effect of norepinephrine by Rall and West.\textsuperscript{142} This raises the question of whether the positive inotropic response to the methylxanthines by themselves can be related to cyclic AMP. The answer to this question is not known, but it seems likely that at least part of this response is due to the ability of these agents to increase the level of cyclic AMP in the heart by inhibiting phosphodiesterase. Support for this view comes from the recent demonstration by Kukovetz and Pöch\textsuperscript{85} that imidazole antagonizes the positive inotropic effect of the methylxanthines in the isolated perfused guinea-pig heart. These investigators also found that imidazole could prevent the positive inotropic response to small but not large doses of the catecholamines. These results are exactly what would be predicted if imidazole were to stimulate phosphodiesterase activity in the beating heart, as it has been shown to do in vitro.\textsuperscript{30} Another interesting piece of evidence in favor of this mechanism of action has been reported by Logan and Cotten,\textsuperscript{143} who found that the positive inotropic response to theophylline was reversed by ouabain at low temperatures in the same way as the catecholamine response was. Other similarities between the cardiac effects of the catecholamines and the methylxanthines have been emphasized by others.\textsuperscript{37, 144, 145}

In general, all of the available evidence is at least compatible with the hypothesis that the positive inotropic response to the methylxanthines is mediated by cyclic AMP. The effect would in this respect be similar to that of the catecholamines, except that the increase in the level of cyclic AMP is brought
about by a different mechanism. At the same time there are indications that the methylxanthines may produce at least one additional effect in the heart which may at times obscure the effects of inhibition of phosphodiesterase. The negative inotropic effect of these agents, which is especially prominent when the level of extracellular Ca\(^{++}\) is high,\(^{146}\) and which seems to be more prominent with caffeine than with theophylline,\(^{142}\) is difficult to explain on the basis of phosphodiesterase inhibition. Another manifestation of this “other” effect of the methylxanthines may be the contracture which was noted when these drugs were perfused through isolated rat hearts at low temperatures,\(^{147}\) an effect which was not seen with the catecholamines. Possibly this effect is related mechanistically to the contracture-producing effect of caffeine in skeletal muscle.\(^{148}\) It may be noted that if an additional effect of the methylxanthines in the heart is eventually established, it will come as no surprise.

To illustrate this point, we might consider some of the known effects of caffeine on enzymes involved in liver glycogenolysis. In addition to its well-known effect on phosphodiesterase, caffeine is capable of inhibiting both adenyl cyclase and phosphorylase and also stimulates the activity of phosphorylase phosphatase.\(^{15}\) The effects on adenyl cyclase and phosphorylase may not be important, since they are not prominent at concentrations which inhibit the phosphodiesterase markedly, but the stimulatory effect on phosphorylase phosphatase is pronounced at concentrations as low as 10\(^{-4}\)M.\(^{15}\) This effect may occur in other tissues as well. For example, Hess and associates\(^{149}\) noted that, while a low concentration of theophylline increased the phosphorylase-activating effect of epinephrine in the isolated rat diaphragm and had no effect by itself, a higher concentration reduced the percentage of phosphorylase in the \(a\) form and caused less potentiation of epinephrine than did the lower concentration. These effects of the higher concentration of theophylline most likely resulted from stimulation of phosphorylase \(a\) phosphatase activity.

Our fourth criterion has not been established. The increases in cardiac output and heart rate which occur in dogs and humans in response to the injection of cyclic AMP cannot be regarded as conclusive, because of the possibility that these could have been indirect effects.\(^{102}\) Our inconsistent results with the isolated perfused working rat heart were mentioned previously. Studies with labeled cyclic AMP led to the conclusion that in the rat heart the nucleotide did not accumulate intracellularly to any significant extent.\(^{104}\) (These results stand in contrast to the more recent studies by Gulyassy and Edelman,\(^{150}\) showing that the toad bladder is relatively highly permeable to cyclic AMP.) Recently Kukovetz\(^{151}\) has studied this problem using the isolated perfused guinea-pig heart. He found that in some experiments a positive inotropic response could be obtained by the use of the dibutyryl derivative of cyclic AMP. In those hearts which responded with an increase in force, and only in those hearts, phosphorylase \(a\) levels were also increased. These observations encourage the view that it may yet be possible to define conditions, in at least some species, under which a positive inotropic response to exogenous cyclic AMP can be demonstrated.

In the case of some of the cardiac metabolic effects of the catecholamines, such as phosphorylase activation, our fourth criterion has been satisfied by the use of broken cell preparations.\(^{38,116}\) By the same token, it might be suggested that a similar approach might be useful in the study of the positive inotropic effect. We investigated this possibility, but with uniformly negative results. Despite certain implications to the contrary,\(^{152}\) it is our opinion that conditions suitable for the study of the positive inotropic effect of the catecholamines in broken cell preparations have yet to be developed. Our objections to the experiments of Stam and Honig and their colleagues will be discussed in more detail in a forthcoming publication.\(^{56}\)
The mechanism by which cyclic AMP could act to increase the force of myocardial contraction is unknown at present. The interaction between actin and myosin must be affected ultimately, but a direct effect of cyclic AMP on this system has not been established. Possibly cyclic AMP acts by promoting the movement of calcium across the cell membrane, as has been suggested. However, the mechanisms by which calcium is transported in and out of the myocardial cell could be affected in many different ways, and the action of cyclic AMP might be very complex. In most cardiac preparations the effects of the catecholamines on the rates of tension development and relaxation (and on the overall duration of systole) cannot be distinguished from the effects of an increase in rate and do not occur at all if the interval between beats is sufficiently prolonged. These and other data led Koch-Weser, Blinks, and Berlin to suggest that the catecholamines act by enhancing the formation of whatever effect (called by them "positive inotropic effect of activation" or "PIEA") was responsible for the effect of increased rate. The fact that these interventions are associated with an increase in the rate of tension development while at the same time causing an earlier onset of relaxation has been referred to as paradoxical, but this is an inappropriate word to use in this case. A paradox is that which is unexpected on the basis of previous knowledge. The fact is that we have hardly any knowledge at all concerning the intimate mechanisms which are involved in maintaining the force and duration of the heart beat. Perhaps further investigations into the nature of the PIEA will eventually disclose a site of action for cyclic AMP.

The positive inotropic response to the cardiac glycosides differs in almost every detail from the response to the catecholamines, and there is no evidence that cyclic AMP is involved in the former response. Under certain conditions the cardiac glycosides are apparently capable of causing the release of stored catecholamines, which in turn leads to an increase in the force of contraction, but this cannot be the mechanism of action of these agents in general. The cardiac glycosides, unlike the catecholamines, appear to act on the state determining the strength of the rested-state contraction, and do not affect the PIEA. Another positive inotropic agent which we might briefly consider is the fluoride ion, which is of special interest in view of its ability to stimulate adenylyl cyclase in broken cell preparations. It is unlikely, however, that the inotropic effect of this ion can be related to a change in the intracellular level of cyclic AMP. In the first place, fluoride exerts an inotropic effect only at the slowest frequencies of contractions and is inhibitory at normal heart rates. Secondly, the inotropic effect is associated not with an increase in the rate of tension development, but rather with a prolongation of the active state. Finally, an effect of fluoride on cyclic AMP formation in an intact tissue has never been demonstrated. Fluoride had no effect, for example, when it was added to a suspension of washed avian erythrocytes. After hemolysis or cell breakage, however, and in the presence of ATP and Mg++, then fluoride produced its characteristic stimulatory effect. The same phenomenon occurs in the case of brain. A concentration of fluoride which had no effect on cyclic AMP formation in brain slices stimulated adenylyl cyclase maximally when incubated with homogenates. Although the inotropic effects of fluoride and other metabolic inhibitors are of great theoretical interest, it is not likely that the study of these effects will throw much light (at least directly) on the mechanism of the positive inotropic action of the catecholamines.

Finally, in this section, we might mention the effect of acetylcholine. Murad and associates found that in broken cell preparations from dog heart the choline esters were capable of inhibiting the formation of cyclic AMP by up to 30%, and it seems possible, or even likely, that some of the cardiac effects of these compounds may be related to this action. This possibility was discussed previously, and no new information has accumulated.
since. The possible effect of parasympathetic stimulation on the level of cyclic AMP in intact cardiac tissue has still not been measured.

Possible Clinical Implications of Cyclic AMP

In this section we might briefly consider some of the possible clinical implications which are beginning to emerge from research relating to cyclic AMP. While this area has not been extensively explored, certain implications, as we have been able to discern them, can be sketched in broad outline.

Cyclic AMP is obviously of vital importance in the maintenance of health. A reasonable corollary of this, especially considering the numerous effects in which cyclic AMP is known to play a role, is that some disease states may result from malfunctions in the control of cyclic AMP levels. These might include several conditions in which hormone levels seem to be normal but the response of the target tissue to the hormone is diminished or absent.

Diagnosis

In general, the relationship of cyclic AMP to a disease state might be best examined by the same criteria used in studying the involvement of cyclic AMP in a hormone action. Assuming the disease under study was the result of faulty hormonal regulation, the following questions could be asked:

1. Does the adenyl cyclase activity in cell-free preparations of the diseased tissue respond to hormones as it does in normal tissue?

2. Do intact cell preparations of the diseased tissue respond to hormones with a change in the level of cyclic AMP in the same way that normal tissue responds? This criterion, although more difficult because of the problem of obtaining specimens in sufficient quantity, may be more important than the first because of the more physiological condition.

3. What are the effects of the methylxanthines or other phosphodiesterase inhibitors (or activators, when available) on the pathological condition?

4. What are the effects of exogenous cyclic AMP or derivatives on the disease?

Answers to these questions would in large part localize the biochemical lesion in diseases involving cyclic AMP. For example, in conditions caused by inappropriately low cyclic AMP levels, the use of exogenous cyclic AMP or one of its derivatives might repair the defect. The other approaches might then be used to localize the defect further. However, if the lesion occurred at a point in the hormone mechanism distal to cyclic AMP, administration of the nucleotide would be ineffective. In diseases involving abnormally high levels of cyclic AMP, agents capable of either stimulating the phosphodiesterase (for example, imidazole) or inhibiting the hormonal activation of adenyl cyclase might be useful.

Preliminary experiments have indicated that diagnosis of diseases involving cyclic AMP may be feasible. Biopsy material could be used for certain studies, especially with cell-free systems. In addition, Dr. R. L. Scott (unpublished observations) has found that human platelets and leukocytes are endowed with adenyl cyclase activities which are sensitive to hormones and, therefore, that blood samples of 10 to 15 ml might be used. Dr. Scott has also found that the adenyl cyclase activities in some tissues obtained at autopsy were essentially unchanged for up to 6 hours post mortem.

In addition, urinary levels of cyclic AMP and cyclic GMP have been found to be high enough to be readily measured. Not all of the factors which are responsible for the urinary levels of the cyclic nucleotides are understood, but it would appear that a factor in the case of cyclic AMP may be the rate of secretion of parathyroid hormone and possibly of vasopressin. As suggested by Chase and Aurbach, the findings with cyclic AMP may lead to a useful test to differentiate the hypercalcemia of hyperparathyroidism from that of nonparathyroid disorders. An elevated excretion of cyclic AMP would be expected in hyperparathyroidism as contrasted to suppressed excretion in other hypercalcemic states. Clinical studies to test this hypothesis are in progress. In addition, high concentrations of several other hormones appeared to
affect the excretion of cyclic AMP by the rat in preliminary experiments.\textsuperscript{165}  

**Therapy**  

Possible therapeutic approaches involving cyclic AMP might be directed at three levels: (1) the stimulation or inhibition of defective adenyl cyclase activity; (2) the stimulation or inhibition of a defective phosphodiesterase activity; and (3) the bypassing of the cellular mechanisms regulating cyclic AMP levels by providing exogenous compounds which either mimic or inhibit the actions of cyclic AMP on cellular processes.

An example of a disease which might involve a lesion at the level of cyclic AMP is bronchial asthma.\textsuperscript{166} In this instance the use of such agents as isoproterenol and theophylline seems well directed at the basic disorder, which may involve a decreased sensitivity to agents which normally tend to increase cyclic AMP levels in bronchial smooth muscle or a supersensitivity to agents which tend to decrease the level of cyclic AMP, or both.

In addition to the well-documented clinical use of catecholamines and methylxanthines in asthma, some measurements of cyclic AMP in the lung have been made. An epinephrine-sensitive adenyl cyclase system has been identified in subcellular fractions of lung,\textsuperscript{66} and phosphodiesterase is also present.\textsuperscript{26} Recently, caffeine and epinephrine have been found to act synergistically on cyclic AMP levels in rat lung pieces (table 2). While this sort of evidence is unsatisfactory because of the heterogeneity of lung and our lack of information about the cell types in which cyclic AMP levels are changing, it does add some support to the hypothesis that bronchial asthma is due to a maladjustment of the cyclic AMP mechanism in bronchiolar muscle.

In certain other cases the recognition that cyclic AMP is involved may lead to the more rational and widespread use of theophylline and other phosphodiesterase inhibitors as therapeutic agents. For example, Breckenridge and associates\textsuperscript{167} have suggested that theophylline might be usefully employed in the treatment of myasthenia gravis, and clinical studies in this direction will be awaited with interest. It would be desirable to have agents which are more specific than the methylxanthines as phosphodiesterase inhibitors, and efforts to develop such agents are currently being made by several pharmaceutical firms.

Several other disorders which might involve faulty regulation of cyclic AMP levels come to mind. For example, since cyclic AMP represents a key regulator of steroidogenesis, it seems possible that certain endocrine disorders involving adrenal or gonadal dysfunction might be related to a primary defect at the level of cyclic AMP in the defective endocrine gland. One might thus consider the judicious use of phosphodiesterase inhibitors in certain cases of steroid hormone deficiency.

The growing recognition that not all adrenergic \(\beta\)-receptors are identical may lead to the development of a more specific (and possibly more useful) series of adrenergic blocking agents. This may be a mixed blessing, however, since species differences\textsuperscript{168} may make testing difficult. For example, although the methoxamine derivatives are effective in preventing the hyperglycemic response to catecholamines in dogs,\textsuperscript{119} they are not effective in the human.\textsuperscript{92, 168} The receptors in the rat

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**Table 2**

**Effects of Epinephrine and Caffeine on Cyclic AMP Levels in Rat Lung Pieces**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cyclic AMP picomoles/g wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>570</td>
</tr>
<tr>
<td>Epinephrine, 5.5 (\mu)M</td>
<td>2,300</td>
</tr>
<tr>
<td>Caffeine, 1 mM</td>
<td>1,180</td>
</tr>
<tr>
<td>Caffeine, 1 mM</td>
<td></td>
</tr>
<tr>
<td>+ Epinephrine, 5.5 (\mu)M</td>
<td>4,730</td>
</tr>
</tbody>
</table>

* Lungs were removed from rats anesthetized with pentobarbital, placed in 50-ml flasks containing 20 ml of Krebs-Ringer phosphate buffer with 200 \(\mu\)g/ml of albumin, and incubated for 20 minutes at 37 C. The tissue was transferred to fresh media with the appropriate additions and incubated for an additional 20 minutes. The incubation was terminated by dropping the lung pieces into an operating Waring blender containing 18 ml of 0.1 N HCl and purified tritiated cyclic AMP (10,000 dpm). Cyclic AMP levels in the homogenates were measured as previously described.\textsuperscript{23}
liver seem to be similar to those in the human.168, 170 Perhaps in the future this type of knowledge will allow the more efficient use of different experimental animals in drug-screening programs.

Finally, it seems likely that nonhormonal factors may be important in regulating cyclic AMP levels in certain tissues. For example, an effect of potassium on cyclic AMP levels has been observed in guinea-pig brain cortex171 and rat diaphragm.172 Thus, it would appear that a great deal of additional work remains to be done before all of the controls on cyclic AMP are understood and before the relationship of cyclic AMP to health and disease can be clarified.

Concluding Remarks

Evidence has been presented to show that cyclic AMP is a second messenger mediating a variety of hormonal responses, including the positive inotropic response to the catecholamines. Our conclusion that the latter response is mediated by cyclic AMP must remain tentative because consistent inotropic effects in response to exogenous cyclic AMP have not been obtained. However, all of the available evidence, some of which we have reviewed in this paper, is compatible with this hypothesis. Whether it will be possible in the future to devise an isolated heart preparation which will be readily and reproducibly accessible to cyclic AMP and which will at the same time retain its ability to contract and relax remains to be seen.

Although cyclic AMP stands as the only well-established second messenger to date, data supporting such a role for cyclic GMP have been obtained. The existence of this nucleotide in urine has already been mentioned, and recently its formation in cardiac tissue has been studied. One interesting finding which has emerged from these studies is that cyclic AMP and cyclic GMP are apparently produced as the result of separate enzymes, which are affected differently by hormones and other factors. These data will be discussed in detail in a forthcoming publication.165

Acknowledgment

The authors are grateful to Dr. Kukovetz151 for a personal communication concerning his most recent research in this area.

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Circulation. 1968;37:279-306
doi: 10.1161/01.CIR.37.2.279

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