ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 185

December 3, 1971

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Publication of this monograph was aided by contributions from:

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CYCLIC AMP AND THE FUNCTION OF EUKARYOTIC CELLS: AN INTRODUCTION

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The chemical structure of adenosine 3′,5′-monophosphate, otherwise known as cyclic AMP, is shown in FIGURE 1. It was discovered as the mediator of the hepatic glycogenolytic effect of epinephrine and glucagon,[1] and is now recognized as a versatile regulatory agent mediating a host of hormonal effects.[2] Research in this area is expanding at such a rate that it is very difficult, if not impossible, for a single group to keep track of it all. Our hope for this present monograph was that it would update and supplement an earlier one,[2] which we hope will continue to be useful as a source of background material. Both of these hopes will probably be realized to some extent, but the incredible pace of research in this area cannot be overemphasized. We are writing these words in October, 1970, with the conference less than a month away, and we fully expect that they will have a faintly old-fashioned ring to them by the time they are in print. Perhaps by extrapolating from what is said here and in the rest of this monograph, the serious reader will gain at least some idea of where the subject is likely to lead in the future.

The structure of adenyl cyclase is still poorly understood. Information about this enzyme was summarized previously,[2-4] and an important recent development is discussed in this monograph by Lefkowitz *et al.*[5] Still more recently, Rodbell and his colleagues [6, 7] have made some useful contributions. There is no longer any question that the receptors for some hormones are very closely related to the adenyl cyclase system as a whole, but most of the details remain to be worked out. We still do not know how the hormone-receptor interaction leads to a change in the catalytic activity of the enzyme. To whatever extent our earlier model (FIGURE 2) represents an aspect of reality, it would appear that the catalytic and regulatory subunits do not necessarily develop at the same time. A more operationally correct way of summarizing the available data [8, 9] would be to say that the catalytic activity of adenyl cyclase and its ability to be stimulated by hormones do not necessarily develop at the same time. There is now evidence that these two components of the system can be separated,[10] and further work along these lines will be watched with interest. The suggestion that GTP or GDP may play an important role in the effects of hormones on adenyl cyclase [7] has also been of interest, but whether future research will substantiate or invalidate this hypothesis remains to be seen.

The detrimental effects of cell breakage on adenyl cyclase, and especially on its sensitivity to hormonal stimulation, were emphasized in an earlier review.[3] However, we can now see that this may be even more variable than it was then thought to be. Platelet adenyl cyclase, for example, seems to be just as sensitive to stimulation by prostaglandins in broken cell preparations as it is in intact cells, as noted by Krishna and his colleagues.[11] Brain cyclase stands at the other end of the scale, with most other cells and tissues falling some-

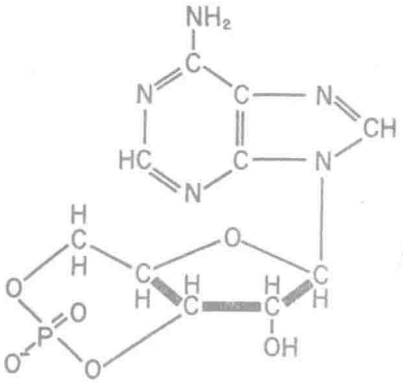

FIGURE 1. Chemical structure of cyclic AMP.

where in between.[3] The reasons for these differences have yet to be investigated in any kind of detail. We can note that platelet membranes appear to contain an unusually large amount of unionized calcium, apparently bound in some manner to membrane phospholipids,[12] but whether this is an important factor contributing to the stability of platelet adenyl cyclase remains to be seen.

The nature of the fluoride effect is also still mysterious. Fluoride has been found to stimulate adenyl cyclase activity in preparations of most eukaryotic cells and tissues studied,[1] and there is some evidence to suggest that this in fact represents the reversal of an inhibitory influence which is not always present and which can be removed.[9, 13, 14] Fluoride has not been clearly shown to alter the level of cyclic AMP in an intact cell, and the reason for this is still unknown.

The phosphodiesterase, discussed in more detail in a following paper,[15] is still the only enzyme (or enzymes, if that is the case) known to be involved in the metabolism of the cyclic nucleotides. Although it has not been established as an important site of hormone action, there is suggestive evidence that alterations in phosphodiesterase activity may be involved in certain instances of feedback regulation. The feedback regulation of cyclic AMP levels is, in

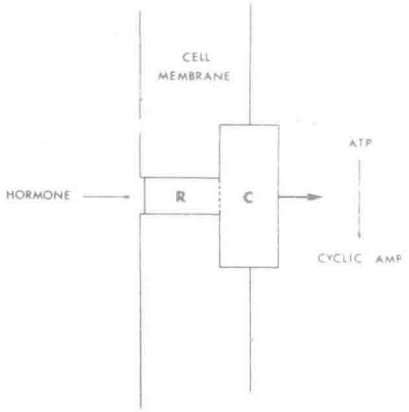

FIGURE 2. Model of the protein component of the membrane adenyl cyclase system. R, regulatory subunit; C, catalytic subunit.

general, an area in which more work needs to be done. Possible metabolic pathways other than conversion to 5′-AMP also need to be considered; the inhibitor studied by Murad and his colleagues [16] may very well be the product of one such pathway. It also seems possible that some of the paradoxical effects of exogenous cyclic AMP (paradoxical in the sense that they are the opposite of the effects of hormones that increase the endogenous formation of cyclic AMP, even though these effects may be accurately mimicked by derivatives of cyclic AMP) [17, 18] may reflect primarily conversion to an as yet unidentified antimetabolite of cyclic AMP. It is important to note, in this regard, that the mechanism or mechanisms by which several hormones act to cause a fall in the level of cyclic AMP have still not been defined.

Considerable recent progress has been made toward understanding the mechanism of action of cyclic AMP. The earlier work by Krebs and his colleagues has been extended by them and by others, as discussed in this monograph [19–22] and elsewhere.[23–27] Whether all of the physiologically important effects of cyclic AMP can be understood as the result of protein kinase stimulation remains in doubt, although this continues to be an attractive hypothesis. We can point to the two papers in this monograph by Lefkowitz and associates [5] and Garren and associates [22] as possibly the most complete description currently available of the mechanism of action of a hormone, in this case ACTH. Although progress has been substantial, obviously much remains to be learned.

A detailed discussion of the mechanisms by which permissive hormones, such as steroids, thyroxine, and growth hormone, may influence the formation or action of cyclic AMP was not on the agenda for this conference. It is clear that many of the effects of these hormones may be related to cyclic AMP in one way or another,[28–31] but details, on the one hand, and general principles, on the other, are still obscure. We anticipate many interesting developments in this area in the future, and some of them will probably have occurred before these words are printed. An interesting series of studies in this regard have been initiated by Weiss and his colleagues as discussed in this monograph.[32]

Rasmussen [33] has attempted to link the calcium ion with cyclic AMP in a unified theory of hormone action. However, the relationships between these two cell constituents seem quite complex. Calcium ions are clearly required in order for some of the effects of cyclic AMP to be expressed, as discussed in this monograph, for example, by Selinger and Schramm.[34] In certain other systems, such as smooth muscle [2, 35–37] and platelets,[38] the effects of Ca^{++} and cyclic AMP are just as clearly opposed to one another. Even such a seemingly similar response as exocytosis, which appears to require Ca^{++} in all cases studied, may be stimulated by cyclic AMP in some cells [34] and inhibited in others.[39] In some tissues, notably skeletal tissues,[40] cyclic AMP may mediate the effect of a hormone on the state of tissue calcium. Conversely, Ca^{++} may be required for some hormones, such as ACTH, to stimulate adenyl cyclase, whereas other hormones, such as the catecholamines, do not have this requirement.[41] Our own tentative conclusion is that more research will be required before these and other observations relating to cyclic AMP and Ca^{++} can be fitted into any kind of general theory. The most positive statement that can be made at present, in our estimation, is that calcium ions seem to be required for the operation of a great many basic processes, some of which are subject to the regulatory influence of cyclic AMP.

Studies involving unicellular organisms were not included on the agenda

of this conference. This decision could only be justified on the grounds (established previously at a Gordon Research Conference) that not all aspects of the subject could be covered in the time available. Studies on the role of cyclic AMP in the regulation of gene expression in bacteria have been reviewed by Pastan and Perlman.[42] An interesting development since that review was published has been the demonstration that cyclic AMP is required in at least some bacteria for flagella formation and hence motility.[43] A cyclic AMP-binding protein is necessary for the effect on mRNA synthesis,[23, 24] but the relation of this protein to the protein kinase previously identified by Kuo and Greengard [44] is still questionable at the time of this writing. Turning briefly to the cellular slime molds, it now appears that at least one other compound besides cyclic AMP can function as an acrasin in (or between) these organisms.[45] It will obviously be of great interest to identify this compound chemically and to see if it also occurs in higher organisms.

At least as distressing to some readers as the omission of the microbiological work will be the lack of emphasis on clinical studies. This area is still in its infancy, but we anticipate many exciting developments in the years to come. It would not be surprising, in our view, if a great many common disorders in humans were ultimately found to be related to defects in the formation or action of cyclic AMP. We hope that most if not all of the information summarized in this monograph will be interesting and useful to clinical investigators. We can call special attention to the review by Broadus and colleagues,[46] which is the most comprehensive review currently available of studies dealing with extracellular cyclic nucleotides.

In concluding these introductory remarks, we will simply express our hope that the conference will have been useful to those who participated in it, and that this monograph will be useful to them and to all other serious investigators. Even if future progress is as rapid as we anticipate, this monograph should stand as a useful guidepost for many years to come.

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METHODS OF ASSAY OF CYCLIC NUCLEOTIDES

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Significant discoveries often have originated in troublesome inconsistencies in analytic procedures. The drive to develop new methods in the measurement of cyclic nucleotides has been sustained by difficulties with existing procedures. A few of these analytic problems have led directly to new findings or interpretations of cyclic nucleotide function. Although this theme is developed most readily by discussing the measurement of cyclic AMP in tissue, it could also be supported by considering the anomalies in measurements of cyclic GMP and of the enzymes of cyclic nucleotide metabolism.

Recently, several fundamentally new procedures and refinements of existing methods have been introduced. Some possess many desirable attributes, but all retain some limiting features. The amount of tissue or body fluid available for study, the skills that the analyst already commands, and the availability of specialized equipment continue to influence the choice of a method. Valuable information for assessing methods has recently appeared in reviews and symposia.[1-4] Rather than attempting to compile a resume of procedures or to judge finally the performance of all available methods, I shall summarize a few instances from the initial struggles to measure cyclic AMP in tissue that clearly led to new insights into its biologic role. Subsequently, I shall attempt to identify some of the features of recently developed procedures that could conceivably provide guides to new discoveries in cyclic nucleotide metabolism.

Initially, Rall and Sutherland [5] estimated cyclic AMP by means of a procedure that was essentially a test-tube bioassay. Liver particles responded to the addition of cyclic AMP by converting inactive phosphorylase to the active enzyme through intermediate steps whose numbers and mechanisms were unknown. It is remarkable that so much reliable information was obtained by means of an assay that exhibited a sigmoid response over a threefold range of cyclic AMP concentration and whose sensitivity was less than that required for the measurement of endogenous levels of cyclic AMP. Furthermore, it was soon recognized that various materials interfered with the assay in either a positive or a negative way. An important observation was that the apparent amount of cyclic AMP in adenyl cyclase reaction mixtures decreased as the size of the sample assayed increased. This has led, through a series of painstaking studies by Murad and associates,[6] to the finding that virtually all mammalian tissues are capable of synthesizing a phosphodiesterase-sensitive inhibitor of the liver particle system. Neither the structure nor the physiological role of the cyclic nucleotide inhibitor has yet been defined, but there seems little doubt of its reality and its potential importance in understanding cyclic nucleotide metabolism.

There is a second example in which an explicit concern with interfering substances in the liver particle assay appears to have contributed to the discovery of an important phenomenon. The liver particle system was greatly improved

by separating cyclic AMP from most other components of tissue extracts by means of ion exchange resins.[7, 8] In the course of that work ADP was identified as one of the interfering materials. Understanding of that fact doubtless influenced the decision in Rall's laboratory to explore the possibility that noncyclic adenine nucleotides of brain extracts accounted for the observed stimulation of brain adenyl cyclase.[9] The function of adenosine receptors in the brain adenyl cyclase system, like that of Murad's inhibitor, remains to be elucidated. They represent a quantitatively impressive phenomenon that is also perplexing because adenosine inhibits adenyl cyclase in broken cell preparations of liver.[10]

A procedure based upon the capacity of cyclic AMP to activate phosphorylase from muscle was initially devised by Posner and coworkers,[11] and later refined by Namm and Mayer.[12] The muscle phosphorylase activation by cyclic AMP also exhibited susceptibility to a naturally occurring inhibitor. This has been identified as a heat-stable, trypsin-sensitive protein, but its physiological role has not yet been determined.[13]

During the use of the phosphorylase-activating systems, analytic inadequacy again served to underscore an important facet of cyclic AMP metabolism. The fault was not in the analysis itself but in the process of fixing and extracting tissue. Divergent results on the effect of epinephrine on cyclic AMP in the heart were explained by the fact that the anoxic interval between biopsy and freezing of cardiac muscle permitted cyclic AMP to disappear.[12] Earlier it had been noted that brief anoxia of brain doubled cyclic AMP levels.[14] Numerous additional examples of major changes in cyclic nucleotide content within seconds are now known. Means of minimizing artifactual changes have been recently reviewed.[4] Limitations of the barium treatment of tissue extracts occasionally ignored are that barium hydroxide catalyzes cyclic AMP formation, precipitates 30–40% of cyclic GMP,[4] and leaves in solution substances that chromatograph near cyclic AMP.[15]

The first method for the measurement of cyclic AMP in tissue that differed in principle from the phosphorylase-activating system appeared in 1964.[14] Cyclic AMP was converted to 5′-AMP by means of purified phosphodiesterase and then to an equivalent amount of ATP through reactions catalyzed by myokinase and pyruvate kinase. The system for detecting ATP utilized coupled reactions catalyzed by hexokinase and pyruvate kinase so that an amount of glucose-6-phosphate several thousandfold greater than the amount of ATP was produced. Refinements of the system devised by Goldberg [4, 16] have permitted reliable measurements at the level of 5×10^{-14} moles of cyclic AMP. Other investigators have utilized the initial reactions of this sequence but have combined them with alternate means of detecting ATP. For example, in the Aurbach and Houston procedure [17] the ATP is measured using $^{32}P_i$ and the exchange reaction catalyzed by phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase. Adenosine triphosphate is estimated by Kaneko and Field [18] by the enzymatic production of $^{14}CO_2$ from labeled glucose-6-phosphate resulting from the coupled reactions catalyzed by hexokinase and pyruvate kinase. The luciferase method is being used as an ATP detector by Johnson and colleagues [19] and by Ebadi and associates.[20] Each of these methodologic variations is capable of reliably detecting cyclic AMP in a few milligrams of tissue, but each requires a quantitative separation of cyclic AMP from other adenine nucleotides. The specificity for cyclic AMP is rigorous and the sensitivity is limited ultimately only by adenine nucleotides bound to the analytic enzymes. In practice, the time for preliminary

separation of cyclic AMP and the requirement for meticulous attention in the use of enzymes as analytic reagents have also been limitations.

For the aforementioned reasons, the initial response to the isotope dilution method of Brooker [21] was one of great hope, but also of considerable skepticism. In any event the development of the isotope dilution method provides a clear illustration of how scrutiny of an analytic procedure can lead to new insights into cyclic AMP metabolism. This operationally simple method is based upon competition between labeled cyclic AMP and nonlabeled cyclic AMP from tissue for the hydrolytic site of phosphodiesterase of brain. Skepticism arose because the apparent K_m of rat brain phosphodiesterase for cyclic AMP was known to be 2×10^{-4} M. Therefore, the effect of summing concentrations of labeled and nonlabeled cyclic AMP, each of which was much less than 10^{-4} M, would be to increase velocity, with little alteration in the amount of labeled cyclic AMP hydrolyzed. Only if a phosphodiesterase were present in brain with an apparent K_m of the same order as the cyclic AMP concentration would one expect dilution of the labeled nucleotide by the nonlabeled material to be apparent. In fact, detailed study of phosphodiesterase activity in brain revealed not only a second enzyme with an apparent K_m of 10^{-6} M, but also a phosphodiesterase relatively specific for cyclic GMP.[22] In addition, the theoretical basis of the observed isotope dilution has been provided.[23]

The study of cyclic AMP in intact tissue has been greatly facilitated [24–27] by the simple expedient of labeling intracellular ATP by means of adenine-^{14}C. Furthermore, in validating this new method as a relative measure of cyclic AMP levels in tissue and in comparing its results with those of earlier studies, several investigators have obtained important evidence for intracellular compartmentation of cyclic AMP metabolism. Following application of hormones and drugs to a variety of tissues, relative changes in cyclic AMP were observed that were usually comparable to those obtained by measuring absolute amounts of cyclic AMP. However, data from Field's recent study of cyclic AMP metabolism in thyroid [28] illustrate the fact that larger increments may be seen by means of the labeled adenine method. Additions of prostaglandin A_1 and prostaglandin E_1 increased the labeled cyclic AMP of dog thyroid slices fourfold, but increased the absolute amount of cyclic AMP only twofold. In addition it has been established by Creveling's group that the specific activity of newly synthesized cyclic AMP in brain slices is invariably higher than the specific activity of the total intracellular ATP pool.[29] It is clear that less than 10% of intracellular ATP is preferentially labeled by adenine-^{14}C and that up to 40% of that restricted nucleotide pool can be used for cyclic AMP synthesis.

The protein kinase methods of Wastila and colleagues [30] and of Kuo and Greengard [31] are derived directly from the discovery of the requirement for cyclic AMP by phosphorylase kinase kinase. The fact that proteins other than phosphorylase kinase could serve as substrates and that related enzymes have been found in numerous other tissues facilitated the developments of these methods. The skeletal muscle protein kinase procedure provides the attractive features of no requirement for purification of acid extracts of tissue, of high sensitivity permitting analysis of a few milligrams of tissue, and of operational simplicity.[30] Dozens of samples can be analyzed in a single day. $^{32}P_i$ incorporation into protein is linearly dependent on cyclic AMP.

The development of the cardiac muscle protein kinase method illustrates well that resolution of problems in the measurement of cyclic nucleotides may have significance beyond the immediate goal of devising an improved analytic proce-

dure.[31, 32] Protein kinases from heart, kidney, and brain were compared and each exhibited a two-phase stimulation by cyclic AMP. The sensitivities of the heart and kidney protein kinases are comparable to that of the muscle phosphorylase kinase kinase (approximately 2 pmoles result in doubling of blank $^{32}P_i$ incorporation), but the brain enzyme is about tenfold less sensitive. Also, in examining the properties of the brain protein kinase with regard to suitability as an analytic enzyme, Kuo and coworkers [32] found that the kinetic behavior of the activated enzyme differed in an important way from that of virtually all other tissues. The concentration of ATP required for half-maximal velocity is lowered ten- to 15-fold by cyclic AMP in the case of the brain enzyme in contrast to others that show little change. It is attractive to suppose that the brain enzyme differs from those of other tissues in order to serve some particular biologic function.

In the cardiac muscle protein kinase method, sensitivity is ultimately limited by the presence of activity in the absence of cyclic AMP. Could this be explained by the occurrence of two protein kinases, one dependent upon cyclic AMP and one independent? Two such enzymes have recently been identified in liver.[33] Another possibility is that a variable amount of endogenous cyclic AMP might be bound to the protein kinase, resulting in its partial activation. It is obvious that questions that arise upon examining a new assay are virtually synonomous with the most compelling current questions about the mechanism of action of cyclic AMP.

Gilman's recent publication [34] of a method for cyclic AMP measurement based upon binding to skeletal muscle protein kinase contains many features that invite further investigation. Cyclic AMP in trichloroacetic acid extracts of tissue competes with tritium-labeled cyclic AMP for binding to muscle protein kinase. Inclusion of the heat-stable protein that inhibits protein kinase actually increases the affinity of the enzyme for cyclic AMP and stabilizes the binding. The protein is adsorbed to cellulose acetate filters that are placed directly in vials for scintillation counting. The extraordinary simplicity of this procedure is apparent, and sensitivity and specificity are claimed sufficient to use milligram-sized portions of tissue. A related procedure [35] that employs a partially purified cyclic nucleotide binding protein of adrenal differs in that a preliminary isolation of cyclic AMP from acid extracts is required and sensitivity is approximately 200-fold less than with the muscle protein kinase.

In the muscle protein system neither the mechanism nor the physiological significance of the stabilization of cyclic AMP binding by heat-stable kinase inhibitor is understood. Also, the stability of the cyclic AMP-protein complex appears to be remarkably high. Magnesium ion affects both cyclic AMP binding to protein [34] and the binding of protein to filters.[36] It would be astonishing if other components of tissue extracts are not found to influence these parameters. With a choice of measuring either enzyme activity or cyclic AMP binding to the same protein, the analyst may inquire what relationship exists between nucleotide binding and catalytic activity of the enzyme. One mechanism for cyclic AMP activation of protein kinase is for the nucleotide to bind to an inhibitory subunit of the kinase that then dissociates, leaving an enzymatically active, but cyclic AMP-independent, molecule.[36] These findings from reticulocytes clearly demonstrate that cyclic AMP can bind to a protein that has no kinase activity. To this fact we may add that the cyclic AMP-binding protein of *E. coli* essential for β-galactosidase synthesis also does not exhibit protein kinase activity.[37] Therefore the earlier speculation that all cyclic AMP actions