Methods in
CYCLIC NUCLEOTIDE RESEARCH

Edited by MARK CHASIN
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PREFACE

This volume attempts to collect some of the more important and useful techniques used in cyclic nucleotide research (particularly cyclic 3',5'-adenosine monophosphate), and to present these techniques in so far as possible in such a form that each chapter may be used as a laboratory manual. The abbreviations "cyclic AMP" and "cyclic GMP" will be used throughout this volume to refer to the 3',5'-cyclic monophosphates of adenosine and guanosine, respectively.

In any volume such as this, there will be some degree of crossover between chapters; such overlap may prove useful in this case. For instance, any of the assays for cyclic AMP may be used for measuring the rate of the adenylate cyclase reaction, or protein kinases specifically activated by either cyclic AMP or cyclic GMP have been useful in the assay of these nucleotides. Although two chapters describe preparation of isolated lipocytes, both are included because the preparation techniques differ according to the use for which the cells are intended.

The volume is divided into three basic parts; Assay of Cyclic Nucleotides, Enzymology of Cyclic Nucleotides, and The Use of Intact Cell Systems. The first part includes descriptions of tissue preparation and five different assays for cyclic AMP (and cyclic GMP). The second part includes preparation of liver plasma membranes and lipocyte "ghosts" and assay of adenylate cyclase in these preparations, and the preparation and assay of guanylate cyclase, cyclic nucleotide phosphodiesterase and cyclic AMP- and cyclic GMP-dependent protein kinases. The third part describes two whole cell systems, isolated lipocytes and isolated adrenal cells, and also includes the techniques of prelabeling pools of ATP with radioactive precursors in a variety of tissues.

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July, 1972

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Chapter 1

ASSAY OF CYCLIC AMP

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From its outset, the study of the biological role of cyclic AMP has been greatly hampered by inadequacies in methodology. The most significant problems have been the very low concentration of

this nucleotide in tissue and the rapidity with which its concentration within cells can change, whether being elevated or decreased. For these reasons the fixation and preparation of samples for assay are as critical as the analytical procedure for its determination. In both areas there are significant pitfalls. In this chapter, we shall describe some of the procedures that we have found to be particularly useful for the preparation and analysis of cyclic AMP samples.

I. SAMPLE PREPARATION

Several of the procedures used in the preparation of samples may depend very much on the method of analysis to be used (see Chapter 2, Section I.G). However, in general, sample treatment involves the following steps: fixation, extraction, fractionation and analysis. These steps will be described in this section. Some variations of these steps peculiar to a given assay will be discussed in greater detail the section on the particular assay. A general scheme for sample preparation is shown in Fig. 1.

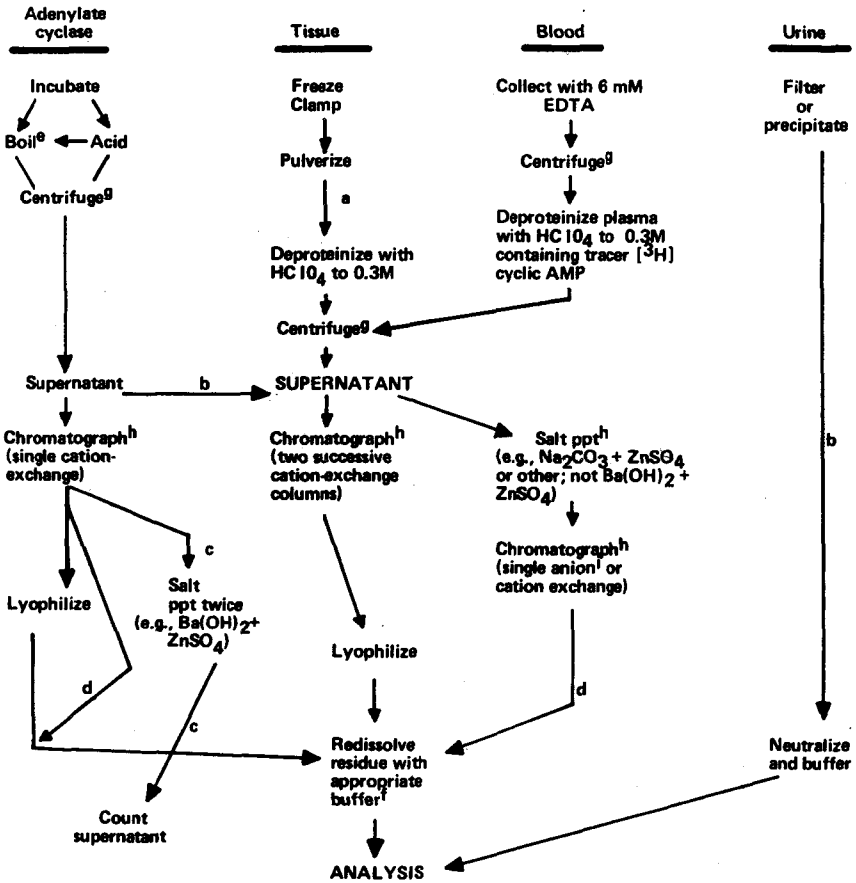

Fig. 1. Scheme for the preparation of samples for cyclic AMP analysis.

[a]tracer [^{3}H]-cyclic AMP added. [b]for luciferase-luminescence assay for cyclic AMP. [c]this sequence can only be used in conjunction with the use of labeled substrate (ATP). [d]lyophilization of eluates can sometimes be avoided if sample cyclic AMP content is high or if

the elution medium is adjusted to elute cyclic AMP in a smaller fraction (e. g., elute with water or buffer). [e]not useful at neutral pH if 5-10 mM Mn^{2+} is present in the incubation. [f]for the luminescence assay, 50 mM glycyl-glycine, pH 7.5; for the protein binding assay, 50 mM Na-acetate-acetic acid, pH 4.0; for the phosphorylase activation assay, 10 mM tris-HCl, pH 7.4. [g]centrifugal force need only be sufficient to sediment denatured protein, etc. [h]see text. [i]if an anion-exchange column is used, 1 mM EDTA should be included in the assay for either the luciferase or binding assay.

A. Fixation and Extraction

Inasmuch as cyclic AMP in tissue has a very high turnover rate, the method of sample fixation is especially critical in tissue studies. Perhaps the most satisfactory procedure for tissue fixation is very rapid freezing (for other methods, see Chapter 2, Section I.G.1). At the appropriate time, the tissue specimen is clamped between relatively massive metal blocks previously cooled in liquid nitrogen. The frozen tissue is then pulverized in a stainless steel impaction mortar, also at liquid nitrogen

temperature. Various procedures have been successfully used for the further fixation and extraction of cyclic AMP from tissue. We have found the following method quite satisfactory. An aliquot of the tissue powder is weighed and then homogenized as rapidly as possible in a motor-driven glass-Teflon homogenizer with about 10 volumes of ice-cold 0.3 M perchloric acid containing about 5000-10000 cpm of tracer [^{3}H]-cyclic AMP per sample for the estimation of cyclic AMP recovery. Trichloroacetic acid and HCl have also been used satisfactorily. The specific activity of the [^{3}H]-cyclic AMP should be as high as possible to preclude assay complications. [^{3}H]-cyclic AMP of about 24 Ci/mmole is comercially available and is adequate for most purposes.

Cyclic AMP in plasma can readily be determined if blood is handled in the following way. Human blood is capable of metabolizing cyclic AMP with an apparent half-life ranging from 30 to 150 min at 37°C [1]. [^{3}H]-cyclic AMP is degraded much more rapidly in rat blood [2] and additional care is necessary. This degradation can be minimized by the reduction of temperature and the addition of methylxanthines or EDTA, and is presumably owing to phosphodiesterase activity. Samples of whole blood are drawn into EDTA

(final concentration 6 mM) [3], mixed and centrifuged at 6000 x g for 5 min at 4°C. Tracer [^{3}H]-cyclic AMP (5000-10,000 cpm per sample) is added to an aliquot of plasma, to which perchloric acid is then added to a final concentration of 0.3 M. Denatured protein, etc., is removed by centrifugation. Deproteinized plasma can be stored as such at 4°C for up to three months without measurable loss of the cyclic nucleotide. Prior to assay, the plasma is chromatographed as described in subsection B.

Assays of cyclic AMP are required also to determine adenylate cyclase activity. In this case, the enzyme reaction is generally terminated by the addition of acid (e.g., HCl to a concentration of 0.1 N), or the sample is placed in boiling water for 2 to 5 min, or both. If adenylate cyclase is being determined by measurement of cyclic AMP formed from unlabeled substrate, it will also be necessary to add tracer [^{3}H]-cyclic AMP to the sample at the time the reaction is terminated so that the cyclic AMP recovery can be estimated (see preceding paragraph). Some further caution should be exercised at this stage. If a solution containing ATP is heated at a neutral or high pH in the presence of certain metals, for example Ba^{2+} [4] or Mn^{2+} [5], cyclic AMP can be formed