HORMONES AND CELL REGULATION

Volume 4

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Edited by:

J. DUMONT and J. NUNEZ

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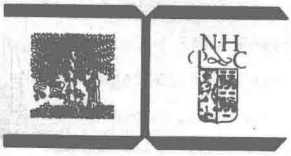

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FOREWORD

During the last few years, substantial progresses have been achieved in the elucidation of systems of cell regulation in superior organisms. In the field of hormone and neurotransmitter action at least three major breakthroughs have greatly stimulated the interest and understanding during recent years : first, these problems can now be studied at the molecular level; second, several hormones are now revealed to act as neurotransmitters; third, all these agents act in different cells on the same fundamental regulation systems. It seems clear now that a limited number of regulation models can account for the main characteristics of such different extracellular signals as hormones, neurotransmitters, ions, etc. All these models imply a primary interaction of the signal with a specific protein, the receptor. Such receptors may belong to the plasma membrane, but also to other subcellular structures. Several types of receptors may correspond to one signal. In any case, the primary interaction of the signal with its receptor leads to a cascade of intracellular events taking place at the level of the plasma membrane, of specific intracellular enzymes, of the protein synthesis machinery, etc. The known physiological responses to the signals are the more or less distal consequences of these cascades.

Those ideas led a group of researchers to organize in 1976, 1977, 1978 and 1979 a four day International Symposium in a small village on the Alsatian side of the Vosges, at the Bischenberg Centre. They believed that it would be of great interest to compare results and concepts derived from studies in widely different areas but bearing all on the mechanisms of cell regulation. Moreover, the need to organize such a meeting at a European level was obvious, as a simple inventory demonstrated the existence of many groups of international reputation, few of which had regular direct contacts, and thus benefited of the cross fertilization of ideas and techniques. This organization was made possible by the help of the "Institut National de la Recherche Médicale" and of the "Délégation Générale à la Recherche Scientifique et Technique" (France).

The meetings have been successfull as they allowed in a relaxed and informal atmosphere to review and discuss recent advances in various fields of cell regulation, which are presented either as scattered and partial communications in general congresses or at lenght in separate specialized meetings. The fact that at Bischenberg, steroid researchers discuss extensively work on cyclic nucleotides, and conversely, is a true measure of the

success of the meetings. Moreover, they have become a very useful forum for European researchers in the field, which attend regularly not only to talk but also to listen and learn. It is the hope of the organizers that they will, in time, constitute a nucleus for European collaboration in the field. These meetings have therefore been organized each year.

The success of a meeting should not imply necessarily the publication of its proceedings. In this case the periodicity of the meetings allows to review regularly recent advances in the various fields of cell regulation by extracellular signals. All authors have been asked to present a rather brief synthetic view of their subject and their research. This should benefit researchers in the same field, but also nonspecialists and students. This book should give them brief and authoritative introductions and syntheses of the state of the art in various fields without having to scan the very dispersed specialized articles. The very rapid publication allows the reviews to be up to date; the predominantly European participation will ensure a European flavor and a fair consideration of European literature which, for various reasons, is sometimes conveniently forgotten. The conferences on fundamental biological problems (eg. membranes, DNA organization, etc.) which have always constituted a significant part of the Bischenberg meeting have not in general been printed in a book which is dedicated to cell regulation. Since this year, methodological seminars are held during the meeting and it has been felt that the moderators of these seminars could, with much profit, write chapters of methodology for the book. These chapters should, year after year, provide up to date and detailed reviews of basic technologies with an effort toward their standardization. The editors have discussed the possibility of editing the discussion of each presentation. However, full publication of the discussion was thought to be both financially and materially difficult and might stiffen the informal character of this discussion. Summaries of the discussion were prepared for Volume 3 but these are open to the criticism that they may reflect more the opinion of the commentator than a true summary. They have therefore been omitted from this volume. Finally the problem of the lecturer who attends the meeting, all expenses being paid, and fails to deliver the promised manuscript had to be faced. In these cases, it has been decided to include in the book a page with the title of the lecture. In summary we would like this book to provide to those who are unable to attend the meeting a good reflection of its scientific information and as to the series, when anybody in need of a short, synthetic and up to date review on cell regulation by hormones or neurotransmitters will turn first to Hormones and Cell Regulation, the editors will think that the endeavour was worthwile.

LIST OF PARTICIPANTS

AHREN K.
Department of Physiology, Fack. S. 400 33 GOTEBORG

AKTORIES K.
Pharmakologisches Institut der Universitat Heidelberg. Im Neuenheimer Feld 366. D.6900 HEIDELBERG

BESANCON F.
INRA, Laboratoire de Biochimie Physique. F.91405 ORSAY Cedex

BLAES N.
INSERM, Unité 63. 22, avenue du Doyen Lépine. F.69500 LYON BRON

BLUNDELL T.
Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, Malet street. GB. LONDON WC1E7H

BOURGEADE M.F.
INSERM, Unité 43. Hopital St Vincent de Paul. 74, ave Denfert Rochereau. F.75674 PARIS CEDEX 14

BOYDS G.S.
Department of Biochemistry, University of Edinburgh, Medical School, Teviot Place. GB. EDINBURGH EH8 9AG

CAILLA H.
Centre d'Immunologie, 70, route Léon Lachamp. F.13288 MARSEILLE CEDEX 02

COOKE B.A.
Royal Free Hospital, School of Medicine, Department of Biochemistry. 8, Hunter street. GB. LONDON WC1N1BP

COUCHMAN J.R.
Colworth Laboratory, Unilerver Limited, Colworth House. GB. SHARNBROOK BEDFORD MK441LQ

DEME D.
INSERM, Unité 96. 78, avenue du Général Leclerc. F.94270 BICETRE

DENTON R.M.
Department of Biochemistry, University Walk, Medical school, GB. BRISTOL BS81TD

DO KHAC L.
Institut de Biochimie. F.91405 ORSAY CEDEX

LE DOUARIN N.
Laboratoire d'Embryologie CNRS, 49bis, avenue de la Belle Gabrielle. F.94130 NOGENT S/MARNE

DROSDOWSKY M.
Laboratoire de Biochimie, CHU. Cote de Nacre. F.14032 CAEN

DROUVA S.
INSERM, Unité 159, 2ter, rue d'Alésia. F.75014 PARIS

DUMONT J.
ULB. Institut de Recherches Interdisciplinaires, Boulevard de Waterloo, 115. B.1000 BRUXELLES

EBEL J.P.
Institut de Biologie Moléculaire et Cellulaire, 15, rue Descartes F.67000 STRASBOURG

EIK NES K.B.
University of Trondheim, Division of Biophysics. N.7034 TRONDHEIM NTH

FELLOUS A.
INSERM, Unité 96. 78, rue du Général Leclerc. F.94270 BICETRE

FRANCON J.
INSERM, Unité 96. 78, rue du Général Leclerc. F.94270 BICETRE

GARNIER J.
INRA. Laboratoire de Biochimie Physique. F.91405 ORSAY CEDEX

GERZER R.
Pharmakologisches Institut der Universitat Heidelberg. Im Neuenheimer Feld 366. D.6900 HEIDELBERG

GEYNET P.
INSERM, Unité 99. Hopital Henri Mondor. F.94010 CRETEIL

GOURDJI D.
Collège de France, 11, Place Marcellin Berthelot. F. 75231 PARIS CEDEX 05

HAGUENAUER-TSAPIS R.
INSERM, Unité 99. Hopital Henri Mondor. F.94010 CRETEIL

HAMPRECHT B.
Max Planck Institut fur Biochemie D.8033 MARTINSREID BEI MUNCHEN

HARBON S.
Institut de Biochimie. F.91405 ORSAY CEDEX

HARDMAN J.G.
Department of Physiology, Vanderbilt University, School of Medicine. USA. NASHVILLE 37232

HEPP K.D.
Institut fur Diabetesforchung. Kolner Platz 1. D.8000 MUNCHEN 23

HOMBURGER V.
Collège de France. 11, place Marcellin Berthelot. F.75231 PARIS CEDEX 05

HOUDEBINE L.M.
CNRZ/INRA, Laboratoire de Physiologie de la Lactation. F.78350 JOUY EN JOSAS

IYNEDJIAN P.S.
Institut de Pharmacologie, Rue du Bugnon, 21. CH.1011 LAUSANNE

JACQUEMIN C.
Laboratoire de Biochimie. Faculté des Sciences. B.P. 347. F.51100 REIMS

JAKOBS K.H.
Pharmakologisches Institut der Universitat Heidelberg. Im Neuenheimer Feld 366. D.6900 HEIDELBERG

JARD S.
INSERM, Collège de France. 11, Place Marcellin Berthelot. F.75231 PARIS CEDEX 05

JEANRENAUD B.
Laboratoire de Recherches Médicales. Avenue de la Roseraie. CH.1211 GENEVE 4

KERAVIS T.
Université Louis Pasteur, Laboratoire de Pharmacodynamie. 74, Route du Rhin. F.67400 ILLKIRCH-GRAFFENSTADEN

KIRK C.J.
Department of Biochemistry. University of Birmingham. P.O. Box 363. GB. BIRMINGHAM B152TT

KORDON C.
INSERM, Unité 159. 2ter, rue d'Alésia. F.75014 PARIS

KRAM R.
Departement de Biologie Moléculaire, Rue des Chevaux, 67. B.1640 RHODE ST GENESE

LACOMBE M.L.
INSERM, Unité 99, Hopital Henri Mondor. F.94010 CRETEIL

LAMBERT M.
ULB. Laboratoire de Chimie Biologique. Boulevard de Waterloo, 115. B.1000 BRUXELLES

MARVALDI J.
Institut de Chimie Biologique. Place Victor Hugo. F.13331 MARSEILL CEDEX 03

MERKELBACH U.
Unité de Recherches Thyroidiennes. Hopital Cantonal. CH.1211 GENEVE

DE MEYTZ P.
Laboratoire de Pathologie Générale. 7529, avenue Hippocrate 75. B.1200 BRUXELLES

MOREL F.
Collège de France. 11, Place Marcel-lin Berthelot. F.75231 PARIS CEDEX

MORERA A.M.
INSERM, Unité 162. Hopital Debrousse. F.69322 LYON

NUNEZ J.
INSERM, Unité 96. 78, rue du Général Leclerc. F.94270 BICETRE

PAUL D.
Temple University. Fels Research Institute. School of Medicine. USA. PHILADELPHIA 19140

PAVLOVIC-HOURNAC M.
INSERM, Unité 96. 78, rue du Général Leclerc. F.94270 BICETRE

RINTERKNECHT E.
Laboratoire de Biologie Générale. 12, rue de l'Université. F.67000 STRASBOURG

ROSSELIN G.
INSERM, Unité 55. 184, rue du Fg Saint Antoine. F.75571 PARIS CEDEX 12

SAEZ J.M.
INSERM, Unité 162. Hopital Debrousse. 69322 LYON CEDEX

SCHULTZ J.
Universitat Tubingen. Pharmazeutiches Institut. Auf der Morgenstelle, 8. D.7400 TUBINGEN 1

SCHULTZ G.
Pharmacologisches Institut der Universitat Heidelberg. Im Neuenheimer Feld 366. D.6900 HEIDELBERG

SELINGER Z.
Department of Biological Chemistry. Hebrew University of Jerusalem. I. JERUSALEM

SCHOWCH G.
Physiologisch-Chemisches Institut. Universitat Krankenhaus Eppendorf Martinistrasse 52. D.2000 HAMBURG

SUTTER-DUB M.T.
Laboratoire de Physiologie Animale UER de Biologie. Avenue des Facultés. F.33405 TALENCE CEDEX

SVOBODA M.
ULB. Laboratoire de Chimie Biologique. Boulevard de Waterloo 115. B.1000 BRUXELLES

SWILLENS S.
ULB. Boulevard de Waterloo 115. B.1000 BRUXELLES

TOUGUI Z.
Institut de Biochimie. F.91405 ORSAY CEDEX

VAN DER MOLEN H.
Department of Biochemistry II. Erasmus University. PO. Box 1738. NL. ROTTERDAM

VAN DE VEERDONK F.C.
Department of Zoology. State University of Utrecht. Transitorium Padualaan 8. NL. UTRECHT

VAN HEYNINGEN S.
Department of Biochemistry. University of Edimburg. Medical School. Teviot Place. GB. EDIMBURG EH89AG

VAN SANDE J.
ULB. Institut de Recherches Interdisciplinaires. Boulevard de Waterloo 115. B.1000 BRUXELLES

VASSART G.
ULB. Institut de Recherches Interdisciplinaires. Boulevard de Waterloo 115. B.1000 BRUXELLES

WALAAS O.
University of Oslo. Institute of Medical Biochemistry. PO. Box 1112, Blindern. N. OSLO

WIRTH A.
Klinikum der Universitat Heidelberg. Bergheimer Strasse 58. D.6900 HEIDELBERG

DE WULD H.
Faculteit der Geneeskunde. Afdeling Biochimie. Campus Gasthuisberg. Herestraat. B.3000 LOUVAIN

CONTENTS

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ANTIBODIES AGAINST CYCLIC AMP, CYCLIC GMP AND CYCLIC CMP. THEIR USE IN HIGH PERFORMANCE RADIOIMMUNOASSAY.

HELENE L. CAILLA, DANIELE ROUX, HENRI KUNTZIGER* and MICHEL A. DELAAGE

Centre d'Immunologie INSERM-CNRS - Marseille-Luminy - Case 906
13288 Marseille cédex 2 - France
*Centre Hospitalier du Luxembourg - Service de Médecine Interne - Département de Néphrologie - 4, Rue Barblé - Luxembourg.

INTRODUCTION

With the advent of radioimmunological methods for cyclic nucleotides our vision of the biological processes in which these molecules are involved has become considerably more detailed and realistic. The basic methodology was established by A.L. Steiner et al.[1,2], who obtained highly specific antibodies and synthesized iodinated analogs of cyclic nucleotides. Later H.L. Cailla et al.[3,4,5] showed that cyclic nucleotides could be acylated in aqueous medium and that their succinylation in biological samples increased the sensitivity of the radioimmunoassay by two orders of magnitude. The possibility of using a few thousand cells for assaying cyclic AMP and cyclic GMP has enabled researchers to undertake refined kinetics on samples as small as insects glands or rat adrenal cortex[6], etc.

The effort to further improve the sensitivity and the reliability of cyclic nucleotide radioimmunoassay has never ceased especially in the case of cyclic CMP recently discovered by A. Bloch[7,8]. The aim of this paper is to help researchers starting to set up or to use cyclic nucleotide radioimmunoassay. We have summarized ten years of experience in this field and we have carried out a careful analysis of the factors on which the signal-to-noise ratio of the radioimmunoassay depends. These factors fall into three categories :

- those linked to the antibodies : affinity, heterogeneity, specificity,
- those linked to the processing of the biological samples : extraction, conversion into succinylated or acetylated derivatives.
- those linked to the incubation procedure : separation of free and bound fractions, γ-counting.

Careful attention was paid to substances prone to interfere with the antigen-antibody reaction, especially to salt coming from the acylation step.

As a consequence high-performance radioimmunoassay of cyclic nucleotides was achieved by the selection of antibodies from a large panel of antisera, by the improvements of the acylation process and by the elaboration of a micro-dialysis technique (in the 10 µl range). This allowed us to solve the difficult problem of determining cyclic AMP concentrations in 30 nanoliters samples of tubular fluid taken by micropuncture at different levels of individual rat nephron.

Preparation of immunogen and production of antibodies

2'O succinylated derivatives of cyclic nucleotides. Succinylated cyclic nucleotides are now commercially available from Sigma or Boehringer Mannheim. However, the amount of succinyl cyclic nucleotide (20 mg) needed for the immunogen and the tyrosylated derivative can be easily prepared according to the following protocol which is based on the special reactivity of the 2'hydroxyl group in water. It yields to a quantitative conversion of cyclic nucleotides into 2'O succinyl derivatives which have just to be separated from excess succinic acid.

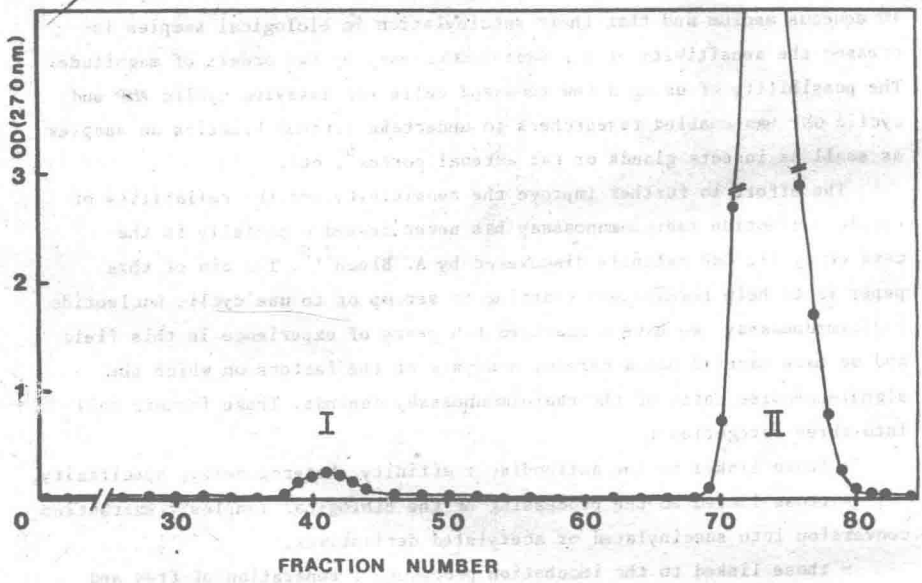

Figure 1. Purification of 2'-O succinyl cyclic CMP on a QAE-A 25 Sephadex column, eluted with a sodium chloride gradient (2 x 0.5 l, 0.02 M to 0.4 M). I : cyclic CMP, II : 2'-O succinyl cyclic CMP.

Twenty five mg of cyclic AMP or cyclic CMP are dissolved in 2.5 ml distilled water alcalinized with 0.175 ml of 9 M KOH. One hundred mg of succinic anhydride are immediately added and the mixture is stirred for 15 min at room temperature. After checking, and eventually adjusting the pH which should be between 6 and 9, the mixture is loaded on a QAE-Sephadex column (10 g), with chloride as counterion. Elution is obtained by a sodium chloride gradient (2 x 0.5 l, 0.02 M to 0.4 M). No buffer is necessary. The peak of succinyl cyclic nucleotide is then loaded on the same QAE-Sephadex column, previously rinsed with water, in order to be desalted. It is eluted with 10^{-2} M HCl, neutralized and lyophilized. A small calculable amount of NaCl remains with the succinyl cyclic nucleotide.

For cyclic GMP and cyclic UMP which are more acidic, the separation of succinic acid from the succinyl nucleotide is achieved by a chromatography on a Dowex 1X2 column (5g, formate form) eluted with a formic acid gradient (2 x 0.5 l, 0.05 N-1N). The succinyl nucleotide peak is pooled and carefully lyophilized.

At the beginning of the synthesis, it is recommended that 100 µCi of tritiated cyclic nucleotide be added in order to facilitate the determination of the ratio hapten/carrier in the immunogen.

Synthesis of immunogen. It is performed in two steps :

1. activation of succinyl cyclic nucleotide by ethylchloroformate in organic medium.

2. coupling of activated succinyl cyclic nucleotide to albumin in aqueous medium.

All operations are carried out at 4°C. About 13 µmole of succinyl derivative are dissolved in 400 µl of dimethylformamide. Sixty µliters of a 1/11 dilution of triethylamine in dimethylformamide and 20 µl of a 1/16 dilution of ethylchloroformate in dimethylformamide are added in that order. The mixture is vortexed and left for 10 min.

Next, a solution consisting of 20 mg serum albumin (human or bovine), 1.5 ml of distilled water and 40 µl of triethylamine diluted to 1/11 in dimethylformamide is added. After leaving the mixture for 15 min at room temperature, it is loaded on a small G-25 Sephadex column (1g) and eluted with 10^{-2} M NaCl. The ratio of fixation is determined at this stage by

analysis of the U.V. spectrum, or better by the radioactivity content if a trace quantity of tritiated nucleotide has been added when preparing the succinylated derivative. After purification, the immunogen may be desalted by dialysis and lyophilized. Eight to 12 moles cyclic nucleotide are fixed per mole of albumin.

Immunization. Any classical immunization protocol can be used. We get good results with the one of Ross et al.[9]. Each rabbit receives a suspension of 0.25 mg to 0.5 mg immunogen with 2.50 mg lyophilized BCG in 1 ml of physiological serum, emulsified with 1 ml of complete Freund's adjuvant. This dose is injected at multiple (about 50) sites into the back of the animals. Booster injections are done at monthly intervals in an identical manner. The course of immunization has to be carefully followed by testing each rabbit once or twice a week. The titer of antiserum is estimated as the dilution able to bind fifty percent of diluted iodinated derivative.

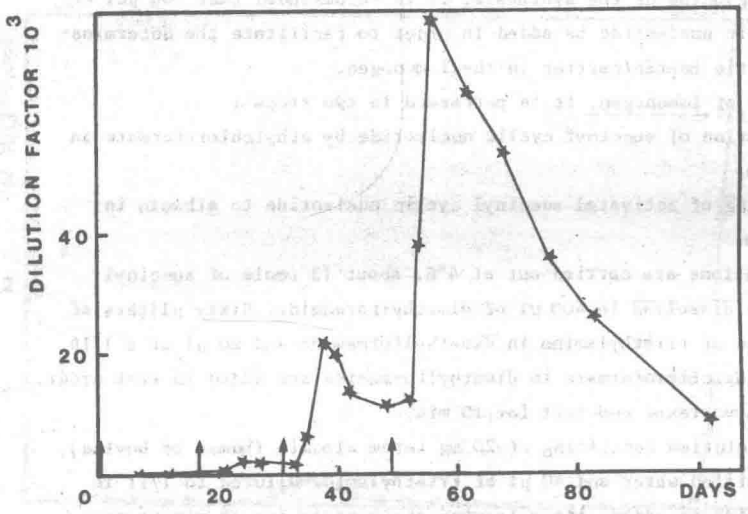

Figure 2. Titer of anticyclic AMP antiserum, as a function of time. Immunogen injections are indicated by arrows.

This roughly corresponds to the ratio of the binding site concentration on the dissociation constant K_d for the hapten antibody equilibrium. A typical profile is shown in figure 2.

Saturation of antibodies by endogenous cyclic nucleotides.

Antibodies produced against cyclic AMP and cyclic GMP are fully saturated by the corresponding cyclic nucleotides[10]. This is due firstly to the high concentration of circulating antibodies (up to 10^{-5} M in binding sites concentrations) as compared to the equilibrium dissociation constant for cyclic nucleotides (around 10^{-8} M), and secondly to the capability of organism to produce cyclic AMP and cyclic GMP at a high rate.

The self-saturation is without importance when using antibodies with iodinated derivatives which are succinylated and thus have a much better affinity for antibodies than cyclic AMP itself. At workable dilution of antiserum the binding site concentration is about 10^{-10} M and endogenous cyclic AMP is no longer bound and does not interfere in radioimmunoassay.

Synthesis of tyrosylated derivative.

The same activation mixture used for coupling a succinyl cyclic nucleotide to albumin can be used for coupling it to tyrosine-methyl ester ; 200 µl of the solution of activated succinyl cyclic nucleotide are added to 100 µl of a solution of tyrosine-methyl ester (15 mg) in dimethylformamide (600 µl) and triethylamine (10 µl).

The reaction mixture is left for 15 min, diluted with 1 ml H_2O and loaded on a small QAE-Sephadex column (0.9 x 20 cm) with chloride as counterion. Elution is obtained by a sodium chloride gradient (2 x 300 ml, 0.02 M to 0.4 M). The tyrosylated derivative is identified by its U.V. spectrum, and by the nitrosonaphtol reaction of phenols. The fractions corresponding to the conjugate succinyl cyclic nucleotide tyrosine-methyl ester are pooled, diluted with a 0.1 M sodium phosphate buffer pH 7.5 to a final concentration of 5×10^{-5} M, divided into 50 µl aliquots and stored at -20°C.

Iodination.

The method is a variation of the original one of Hunter and Greenwood[11], adapted for repetitive iodinations[12]. A solution of chloramine T in dioxane (2 mg/ml) is distributed in Eppendorf tubes into 5 µl aliquots which are

allowed to dry. Each tube is convenient for one iodination with one or two mCi and can be stored for months. Iodination consists in adding 50 µl of tyrosylated derivative, 10 µl of ^{125}INa (carrier free, 100 mCi/ml) into the tube containing chloramine T. The tube is vortexed and left for 2 min. The reaction is stopped by adding 20 µl of a solution of 0.6 mg/ml sodium metabisulfite in water, and 500 µl 0.1 M citrate buffer pH 6.2 with 0.5 g/l sodium azide. The same buffer is used for chromatography of the iodinated derivative on a (0.9 x 60 cm) G-25 Sephadex column which separates the diiodo-, the monoiodo- and the uniodinated derivatives. The elution profile of iodinated derivatives of cyclic AMP is shown in figure 3. The monoiodo derivative has the same specific radioactivity as iodine itself, approximately 1800 Ci/mmole (Radiochemical Center, Amersham). The monoiodo-derivative is diluted (to approximately 3 x 10^5 cpm/ml) in 0.1 M citrate pH 6.2, 0.5 g/l sodium azide, 2 g/l albumin. No loss of immunoreactivity was observed even after 5 months of storage.

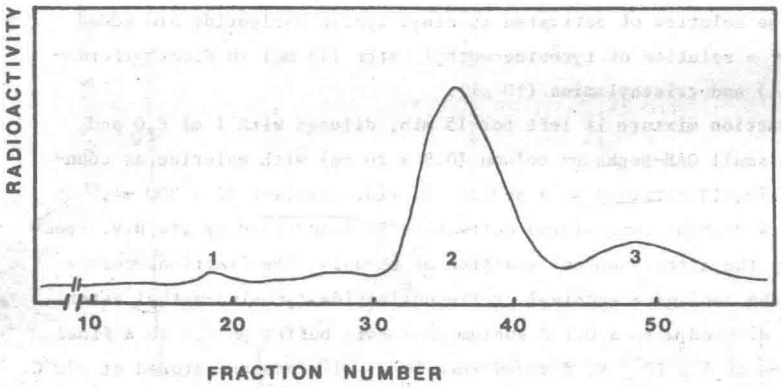

Figure 3. Purification of ^{125}I iodosuccinyl cyclic AMP tyrosine methyl ester. The radioactivity of the effluent is recorded. The volume of a fraction is 3 ml. 1 : iodide - 2 : monoiododerivative - 3 : diiododerivative.