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DETERMINATION OF THE INTRACELLULAR LEVELS OF CYCLIC ADP-RIBOSE IN CULTURED HUMAN CELLS USING A NEW HIGHLY SENSITIVE FLUORESCENT HPLC METHOD Paramjit Kaur Gill*. Department of Molecular Biology and Immunology, University of North Texas Health Science Center at Fort Worth, TX 76107-2699.

Cyclic ADP-ribose (cADPR) is a second messenger that mobilizes intracellular stores of calcium in higher eucaryotic cells. The intracellular concentration of cADPR has previously been estimated to be in the femto- to nanomolar range. Therefore, there is a need for a highly sensitive assay to measure the levels of the nucleotide in just a few million cells. Here, we have developed a highly, sensitive, specific, and reproducible fluorescent HPLC method to determine the intracellular concentration of cADPR in cultured cells. The procedure involves extraction of the nucleotide pool in 20% (W/V) TCA followed by the purification of soluble molecules containing two or more soluble molecules containing two or more riboses by boronate affinity chromatography. Purified nucleotides are then digested with phosphodiesterase to degrade all non-cyclic molecules, leaving cADPR intact. Contaminating products of phosphohydrolysis are then eliminated by a second boronate step and the pure preparation of cADPR obtained is converted to monomeric ADP-ribose with NADase (isolated from *Bungarus fasciatus*). After a third boronate purification, ADP-ribose is chemically derivitized to the etheno-adenine fluorescent form with chloroacetylaldehyde at 60 °C, and the εADP-ribose formed is quantified by fluorescent-HPLC on a Partisil 10-SAX column. The specificity of our method was monitored by determining the yield at every step of the protocol with [^{32}P]cADPR. Radiolabeled cADPR was synthesized from [^{32}P]β-NAD$^+$ and pure ADP-ribosyl cyclase from *Aplysia californica*. [^{32}P]cADPR was subsequently purified by HPLC on a Partisil 10-SAX and a C-18 reverse phase column placed in tandem. While the recovery of a known amount of cADPR

through each boronate step of the 4-day protocol was approximately 90%, the overall recovery throughout the procedure was between 30-40%. As expected, our mock incubations (negative controls) in the absence of phosphodiesterase or NADase treatment, as well as chloroacetaldehyde, yielded no εADP-ribose peak. Furthermore, spiking of a cell extract with commercially available cADPR resulted in the formation of a bigger fluorescent peak. Finally, our method indicated an intracellular concentration of cADPR in HeLa cells of 980 pmol of cADPR/10^8 cells. Considering HeLa cells have a larger cytoplasm compared to blood cells, our results agree well with those reported by Da Silva *et al.* who observed that the intracellular concentration of cADPR was 198 pmol/10^8 Jurkat cells, using a less sensitive chromatographic assay. Applications of this assay as a tool for biochemical investigation as to the role of cyclic ADP-ribose in the signal transduction events of rapamycin as an immunosuppressant and CD38 in B-CLL are discussed.

*The laboratory studies in this thesis were performed entirely in the laboratory of Dr. Rafael Alvarez-Gonzalez, in the department of Molecular Biology and Immunology and were performed entirely under his supervision. Dr. Alvarez served as my mentor from 1995-1998. Both Drs. Rafael Alvarez- Gonzalez and Ronald Goldfarb, Department Chairman, served as co-mentors from August 1999-March of 2000. From April 2001 – July 2001, Dr. Goldfarb served as my mentor.

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Finally, I would like to dedicate this thesis to the "Family." For they believed I could do whatever. They supported, and gave me words of inspiration through this long endeavor. But foremost, I would like to thank God. Waheguru gave me the courage and the strength to help myself when I thought I was broken. It was only through his hand that I was allowed to achieve this.

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ABBREVIATIONS

ADPR, adenosine diphosphoribose;

AG-MP1, analytical grade - MP1;

AMP, adenosine monophosphate;

ADP-ribosyl cyclase, adenosine diphosphoribosyl cyclase;

B-CLL, B-cell chronic lymphocytic leukemia

Ca^{++}, calcium;

CD38, cluster of differentiation 38;

cADPR, cyclic adenosine diphosphoribose;

HPLC, high pressure liquid chromatography;

GDPR, guanosine diphosphoribose;

IP_3, inositol 1,4,5-trisphosphate;

β-NAD^+, β-nicotinamide adenine dinucleotide;

β-NGD^+, β-nicotinamide guanine dinucleotide;

β-NMN, β-nicotinamide mononucleotide;

NAADP, nicotinic acid adenine dinucleotide 2'- phosphate;

Nam, nicotinamide;

NMR, nuclear magnetic resonance;

PR, phosphoribose;

SAX, strong anion exchange;

RyR, ryanodine receptor;

CHAPTER 1

INTRODUCTION

I. PREFACE TO THESIS INVESTIGATIONS

The experimental investigation delineated in this thesis describes a novel, highly sensitive fluorescent HPLC assay for the measurement of cADPR. Whereas this new method is complex and involved, it is also sophisticated and employs current state of the art technology in order to measure the exceedingly minute amounts of cADPR found within the cell. In addition to describing this painstaking methodological development, this thesis describes how this new method can advance the field of cADPR. While prior published methodology lacks accessibility, reproducibility, specificity and / or sensitivity, the method is devised and developed in this thesis is highly accessible, reproducible, specific, and sensitive. This method is therefore of pivotal importance in the area of cADPR research and can now be employed for hypothesis testing of critical biochemical and molecular questions which can yield new mechanistic insights and thus propel new advances in this field of research. In sum, this thesis not only describes a novel, sensitive and reproducible assay which advances the field but represents a tool for further elucidation of mechanistic issues regarding the biochemical functions of cADPR. Several specific examples of how this new method can be used to answer key as of yet unresolved mechanistic questions are provided which clearly show the powerful mechanism resolving potential of this novel technology. The work described in this dissertation is by no means all inclusive of my graduate studies. It places an emphasis only upon key experiments that illustrate the development of the assay to measure cADPR by fluorescence HPLC for fulfillment of a master's degree requirement. In this thesis, I first introduce background regarding Ca^{++}, the

second messenger – cADPR, the enzymes associated with its production and breakdown, the ryanodine receptor, the mechanism of Ca^{++} release via cADPR, and previous work done as a basis used for devising and developing this method. Next, materials and methods for the assay is delineated. A results section following a discussion within which the applications of the method in further studies and importance of cADPR is also provided.

II. CALCIUM MOBILIZATION

a. Role of Calcium in the Cell

Mobilization of intracellular Ca^{++} is a signaling mechanism that is of fundamental importance in the life history of a cell. Normally, basal intracellular cytoplasmic Ca^{++} concentration ranges from 60 – 100 nM and often elevates to *u*M levels in order to initiate various signaling events downstream (5, 6, 10). These include fertilization and many of the developmental processes such as cellular differentiation. It also functions to regulate secretion, contraction, metabolism, proliferation, learning, memory, and finally apoptosis in cellular physiology (5, 6, 10).

b. Calcium Release Activators

i. Inositol 1,4,5-trisphosphate ($InsP_3$)

IP_3 was discovered in 1983 to mobilize Ca^{++} in permeabilized pancreatic acinar cells. Since then, the involvement and mechanism of IP_3 in numerous signaling pathways in many cell systems has been demonstrated (5, 6). In short, IP_3 is produced by PIP_2 hydrolysis within the plasma membrane. IP_3 itself is water soluble and able to diffuse to the cytosol. Here it releases Ca^{++} by binding to IP_3 – gated Ca^{++} release channels in the ER membrane. These channels are regulated by positive feedback. The Ca^{++} released binds back to the channels to increase the Ca^{++} released. In order to stop this process, one of two mechanisms is implemented. Either IP_3

is dephosphorylated or the Ca^{++} entering the cytosol is pumped out. In addition to dephosphorylation, phosphorylation of IP_3 to form IP_4 also occurs. These enzymes are activated by an increase in Ca^{++} levels providing a negative feedback mechanism for IP_3. This illicits a slower yet longer response of the cell or causes the promotion of Ca^{++} stores to be refilled from the extracellular fluid.

Although the mobilization of Ca^{++} was attributed to IP_3, it was noted by many researchers that some remaining Ca^{++} still remained untapped by IP_3 and could be released under certain conditions. The question remained how was that final Ca^{++} mobilized (9)?

ii. Cyclic ADP-Ribose

In 1987 along with other possible candidates for Ca^{++} release activators, nicotinamide adenine dinucleotide, β-NAD^+, were tested in sea urchin egg homogenates and found to mobilize Ca^{++}. Unlike an IP_3 induced immediate response, a short time lapse of 1 to 4 minutes was observed with β-NAD^+(9). In 1989, Lee et al documented that cyclic ADP-ribose was generated from β-NAD^+ by an enzymatic digestion following purification and structural characterization using radioactive precursor labeling, phosphate determination, high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and mass spectrometry (36). As shown in Figure 1, the adenine ring of the β-NAD^+ molecule forms an N-glycosidic linkage with C1' of the terminal ribosyl unit displacing the nicotinamide group to form cADPR.

At this point, cyclic ADP-ribose was shown to be a potent Ca^{++} mobilizing agent. However, very little was known about the enzymes that produced and broke it down, where was it produced, how and how much was produced and the reasons why. These aspects of the biomolecule were and are necessary to answer in order to fully understand the role of cADPR in the cell.

Why did it take nearly four years after IP_3 for cyclic ADP-ribose to be discovered? IP_3 is found to be in picomole to nanomole per mg concentrations whereas cADPR is hypothesized to be in femtomole to picomole per mg quantities within the cell. In addition, relatively larger amounts of IP_3 are needed than cADPR to mobilize the same amount of Ca^{++}. Therefore in order to discover, and then study the molecule, simply the technology for detection had to improve to detect it.

iii. Calcium Pools

Several lines of evidence suggest that IP_3, NAADP, and cADPR-sensitive pools overlap. NAADP, IP_3, and cADPR induced Ca^{++} releases are non-additive (22). There is an inverse relationship between the NAADP, IP_3 and cADPR induced Ca^{++} release (22). Sequential additions of agents to homogenates releasing Ca^{++} illustrate that less was available from the pool for the other to release (22). Percoll density centrifugation causes NAADP, IP_3 and cADPR sensitive microsomes to co-migrate during fractionation (22). These experiments all illustrate conclusively that there are overlapping Ca^{++} pools (17). A Venn diagram illustrates the extent of overlap distribution of agonist sensitive Ca^{++} pools in sea urchin egg homogenates (Figure 2)(22).

III. ENZYMES OF CYCLIC ADP-RIBOSE METABOLISM

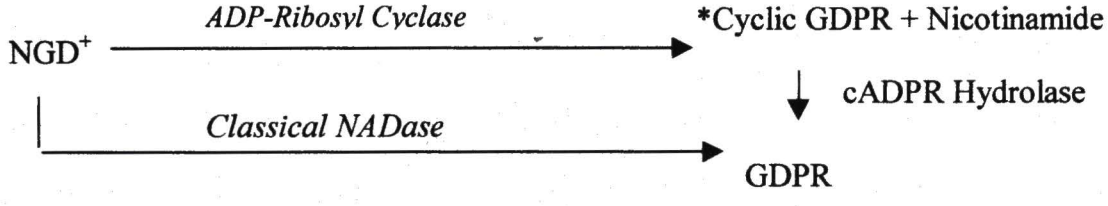
NADases were the first ADPR transferring enzymes discovered over 40 years ago. NAD glycohydrolases are enzymes that convert β-NAD^+ to ADPR by clipping off the nicotinamide moiety. Kim *et al.* (30) were the first to propose a mechanism by which NAD glycohydrolases bifunctionally catalyzed the synthesis of cADPR from β–NAD^+ but also contained hydrolytic activity in which cADPR is cleaved to form the free monomeric ADPR. Figure 1 illustrates the metabolic pathway of cADPR formation and hydrolysis. The cleavage of the nicotinamide –

ribose bond from β-NAD^+ generates an enzyme-stabilized (ADP-ribosyl) oxocarbonium ion intermediate that goes on to produce cADPR. Alternatively, cADPR can react with either the nicotinamide to regenerate β-NAD^+ again or hydrolyze in the presence of water to form an ADP-ribose molecule. The fact that ADPR is not a substrate indicates the cyclase directly utilizes β–NAD^+ to make cADPR instead of going through ADPR as an intermediate step.

Much progress has been made in identifying the metabolic enzymes of cADPR. The synthesizing enzyme, ADPR cyclase, and its degradative enzyme, cADPR hydrolase, have been shown to be ubiquitous in protist (38), plant and mammalian species. ADP-ribosyl cyclases have been reported to be either soluble or membrane bound.

a. Nicotinamide guanine dinucleotide (NGD^+)

In order to study ADP-ribosyl cyclase, Graeff *et al.* (24) was able to utilize β–nicotinamide guanine dinucleotide, β–NGD^+, as a substrate to make cyclic guanine diphosphoribose, cGDPR. Cyclic-GDPR does not have Ca^{++} releasing activity however it is fluorescent, whereas the substrate, β–NGD^+, and the hydrolysis product, GDP-ribose, is not.



Therefore, the cyclase reaction can be monitored by simply measuring the increase in the fluorescent compound. It is also an important tool for distinguishing enzymes involved in cADPR metabolism from classical NAD glycohydrolases. The classical NADases can only produce non-fluorescent GDPR from β–NGD^+ and not cGDPR.

b. Inhibitors of enzymes

i. Adenosine Triphosphate

ATP has been found to inhibit cADPR hydrolase *in vitro* and intracellularly. Takasawa *et al.* (52) illustrated in pancreatic beta cells, that ATP (2-10 mM), generated in glucose metabolism, inhibited the cADPR-hydrolyzing activity, resulting in increased formation of cADPR (52).

ii. ADP-Ribose

The end product of the bifunctional enzyme NAD glycohydrolase, ADP-ribose, was illustrated in sea urchin eggs by Galione et al to decrease cADPR degradation and thus releasing Ca^{++} at higher concentrations (above 100 μM). At lower concentrations (10-100μM) of ADPR exposure to sea urchin eggs inhibits the hydrolysis of cADPR and potentiation of the conversion of cADPR from NAD occurs (21).

iii. NITRIC OXIDE

Nitric oxide (NO) can activate the cADPR pathway (15). A cGMP –dependent pathway leads to the formation of cADPR consequently leading to Ca^{++} release. It was previously shown that microinjection of cGMP into live sea urchin eggs activated Ca^{++} release after a delay. The delay suggested an activation of a cGMP –dependent process leading to Ca^{++} mobilization (13). Exposure of sea urchin eggs to NO, increased cellular cGMP and led to Ca^{++} release. This process could be prevented by the antagonist 8-amino-cADPR to block the receptor or by nicotinamide, an inhibitor of the cyclase (16).

c. Cluster of Differentiation 38 (CD38)

CD38 is a 45-kDa type II trans-membrane glycoprotein. CD38 is a bifunctional NADase that catalyzes both the synthesis and hydrolysis of cADPR. It was originally described as a differentiation marker that has a discontinuous pattern of expression in leukocytes, although

evidence is accumulating of its widespread expression outside of the hematopoietic system. It is found to be widely distributed in terminally differentiated cells, including red blood cells (58) and brain cells (40). Examples of the enzyme include membrane bound enzyme NADases from various spleen preparations, membranes prepared from brain, smooth or cardiac muscle, from total membranes of NG 108-15 neuroblastoma X glioma hybrid cell, and from mitochondrial membranes.

i. Enzymatic Active Site

Recently, the enzymatic active site of CD38 was identified by site directed mutagenesis. E226 was changed to D, N, Q, L, or G and this mutation eliminated essentially all enzymatic activities, indicating that this amino acid is likely the catalytic residue (41).

Other members of the class of membrane bound NADases are CD157 (BST-1), which is expressed on activated T and B lymphocytes, NK cells, erythrocytes and bone marrow stromal cells.

ii. Soluble CD38

Soluble CD38 (sCD38) is also present *in vivo*. Funaro *et al.* assayed normal (fetal serum and amniotic fluid) and pathological (serum and ascites from patients with multiple myeloma, and serum from patients with AIDS) biological fluids to contain the sCD38 (16). Immunoaffinity chromatography, SDS-PAGE and Western blot analyses with monoclonal and polyclonal antibodies, along with metabolic labeling, illustrate a structure of sCD38 to be of 39 kDa (16).

IV. RECEPTOR STUDIES OF cADPR

a. Antagonists of Receptors

Heparin, an inhibitor of IP_3 dependent Ca^{++} release, does not affect cADPR dependent Ca^{++} release therefore suggesting another receptor for cADPR. The presence of nanomolar

concentrations of cADPR totally blocks ^{32}P- cADPR binding in sea urchin egg homogenates while other nucleotides i.e. ATP, ADP, AMP, and cAMP have no effects. Caffeine at millimolar concentrations sensitized cADPR mediated Ca^{++} release.

b. Ryanodine Receptor

The ryanodine receptors are a family of intracellular Ca^{++} channels coded by different genes and recognized to play important roles in the homeostasis of the cation. This calcium channel is a homotetramer in which each monomer is 565 kDa. The pharmacological reagents that modulate the ryanodine receptor are also found to be the reagents responsible for the modulation of cADPR-induced Ca^{++} release. Caffiene is a stimulator of the ryanodine receptor and at high concentrations it releases Ca^{++} from cADPR stores however at lower concentrations it is responsible for potentiating the effect of cADPR. Known antagonists of the ryanodine receptor (i.e. ruthenium red, procaine, and Mg^{++}) completely block cADPR-dependent Ca^{++} release. The effect of ryanodine has been illustrated to be more complex. In sea urchin eggs, ryanodine releases Ca^{++} from the stores of cADPR (19, 31). In other systems such as pancreatic acinar and neurons, ryanodine blocks the action of cADPR. These seemingly opposite effects of ryanodine are hypothesized to be attributable to the biphasic action of the RyR channel (39). Intially, the RyR receptor was thought to be endogenously directly modulated by cADPR. However, later the action of cADPR is found to require accessory proteins. By photoaffinity labeling it was shown that cADPR binds to the 140 kDa protein. Calmodulin is another cofactor involved in the activation of Ca^{++} release by cADPR. Due to these cofactors, the subject of the ryanodine receptor was controversial. Negative results were reported with the RyR due to the fact that the action of the cADPR on the RyR requires accessory proteins and the conditions used to isolate and reconsititute and test the RyR were not present (54, 18, 8).

Evidence supporting the RyR has recently been found in permeabilized PC12 cells and evidence has been correlated with the cell lines devoid of RyR that do not respond to cADPR (13).

c. Analogs of cADPR

Cyclic-ADPR is suggested to be active in the nanomolar range implying a receptor-mediated mechanism for Ca^{++} release. Analogs synthesized of cADPR illustrate the 8-position critical for Ca^{++} release activity. Substitutions of the 8-hydrogen of the adenine ring with a bromo-, amino-, or azido- group all are antagonists and compete with ^{32}P cADPR to bind to the receptor. The inactivation caused by binding illustrates occupation of the receptor is a necessary yet an insufficient condition for Ca^{++} release.

V. ACCESSORY PROTEINS

a. FK506 Binding Protein (FKBP12.6)

FKBP12.6 is a ubiquitously expressed 12,000 – dalton prolyl isomerase. It co-purifies with the ryanodine receptor. It was originally described as the cytosolic receptor for the FK506 or rapamycin. FKBP12.6 is physically associated with the ryanodine receptor, however, in the presence of FK506 and rapamycin the physical association is disrupted (7). Islet microsomes treated with FK506 reduced Ca^{++} release by cADPR. As described in 1997 by Noguchi *et al.*, FKBP12.6 dissociated from the microsomes when treated with cADPR, and moved to the supernatant (43). The microsomes that did not have FKBP12.6 did not show Ca^{++} release by cADPR. These results propose cADPR to be the natural ligand for FKBP12.6 for the ryanodine receptor. Consequently, the binding of cADPR to FKBP12.6 frees the ryanodine receptor from FKBP12.6, causing it to release Ca^{++}.

b. Calmodulin

Calcium release activity of cADPR requires the soluble protein factor calmodulin (CaM) (8, 32). The sensitivity of microsomes to cADPR is altered several orders by magnitude depending upon the concentration of CaM. The requirement of CaM is specific for the cADPR – dependent Ca^{++} release. IP_3 sensitive mechanisms do not show a dependence on CaM.

VII. MECHANISM OF cADPR RELEASE

Within the cell it is currently hypothesized that the mechanism for cADPR action is as follows. ATP competes with cADPR for the binding site of CD38, resulting in the inhibition of the hydrolysis of cADPR and thereby causing cADPR accumulation. CD38 is internalized and cyclic ADPR is released by an unknown mechanism. Cyclic ADPR binds to FK506-binding protein 12.6 (FKBP 12.6) associated with the ryanodine receptor (RyR), dissociating the binding protein from RyR to induce the release of Ca^{++} from the endoplasmic reticulum. Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylates the RyR to sensitize and activate the Ca^{2+} channel. Ca^{2+} is released from the RyR, further activating CaM kinase II and amplifying the process. Thus, cADPR acts as a second messenger for Ca^{2+} mobilization.

VIII. ECTOCELLULAR GENERATION AND INTRACELLULAR FUNCTION

One of the major unresolved questions is the correlation of CD38 ectocellular generation of cADPR and intracellular cADPR induced Ca^{++} mobilizing activity. How ectoenzymes can produce cADPR inside the cell is a topological paradox. Internalization of CD38 to produce cADPR inside has been suggested. NAD induced internalization of CD38 may be related to the known effects of self-aggregation of CD38. If internalization were mediated by vesicular transport, it could lead to exposure of the enzyme to acidic environments such as the interior of

endosomes. Acidic pH is the optimum condition for cADPR production by NADases. However, the mechanism of the CD38 internalization and then release from endocytic vesicles is not clearly delineated.

a. Role of Extracellular cADPR

The ectoenzyme CD38 is present on the outside of the cell and cADPR is produced there also. There are extracellular functions of cADPR that potentiate proliferation of B lymphocytes in response to it. It has been shown in cultured cerebellar granule cells, cADPR and NAD placed exogenously, enhanced the peak of depolarization - induced rise in intracellular Ca^{++} concentration. These effects were seen in a few minutes of cADPR application and thus a time frame within which possible internalization would be minimal.

IX. ASSAYS FOR MEASUREMENT OF CYCLIC -ADPR

Despite its ubiquitous distribution, the precise biological mechanism of action, production and breakdown of this signal transducer to elicit a response within the cell is still incompletely understood. Stimuli that produce changes in cADPR levels are unknown and even quiescent levels are still controversial. The lack of a suitable assay has been a major obstacle in evaluating its role(s) *in vivo* that likely would be obtained by the direct analysis and quantification of the intracellular content of cADPR levels in cellular samples. Therefore, in order to elucidate the precise signaling mechanism of cADPR, a more sensitive biochemical assay is vital.

a. Radiolabeled - cADPR

In order to develop a biochemical assay, a radiolabeled marker is needed for controls to follow the recovery. Since its discovery, biochemical research in this field has been facilitated by the development of radiolabeled - cADPR i.e., in order to develop a radioimmunoassay and

controls for the chromatographic separation of the nucleotide (3, 4, 5). However, the labeled nucleotide as of yet is commercially unavailable. As previously stated, ADP-ribosyl cyclase synthesizes cADPR by converting one mole of β-NAD^+, the substrate, into equimolar amounts of the cyclic nucleotide and nicotinamide. A common procedure for the analysis of synthesized radiolabeled -cADPR is an HPLC method using a strong anion exchange resin AG-MP1 (6, 7). Under these conditions, adenine containing nucleotides are separated using a gradient of increasing concentration of trifluoroacetic acid up to 150 mM at a flow rate of 4 mL / min. With this system, the retention times of Nam, β-NAD^+, cADPR and ADPR have been reported to be 0.66, 1.86, 2.75, and 3.8 min respectively. Unfortunately, cADPR and nicotinamide are separated from β-NAD^+ in a time span of only 5 minutes, and there by, increasing the potential for cross-contamination. In addition, the AG-MP1 column is not commercially available as a pre-packed column, consequently affecting the reproducibility of the system.

Therefore, there is a critical need for a more efficient, sensitive, cost effective and reproducible method to quickly synthesize and purify radiolabeled - cADPR is necessary in order to further facilitate the research in the field.

b. Measurement of cADPR

Earlier reports have measured cADPR levels by a calcium release bioassay, radioimmunoassay, paper chromatography, column chromatography, or by an HPLC assays. Currently, three main methods are used to measure the second messenger, cADPR and are described below.

i. Sea Urchin Egg Homogenate Calcium Release Bioassay

The first is the most commonly utilized sea urchin egg homogenate calcium release bioassay. It bases its measurements of cADPR upon the ability of the tissue extracts to release

Ca^{++} from the reporter - sea urchin egg homogenate preparation. This method is not directly measuring cADPR. It is measuring the Ca^{++} released by cyclic ADP-ribose. There are many pitfalls thus associated with this method. The first being that cADPR Ca^{++} pools overlap with IP_3 and NAADP pools yet are distinct. Therefore in order to see a response, elimination of all other Ca^{++} release activators and/or inhibitors that are present in the sample are needed otherwise they would strongly interfere with the bioassay. In addition, cADPR is thought to be responsible for calcium induced calcium release and could potentially be eliciting a calcium response that in turn is leading to more calcium being released from both intracellular stores and membrane channel involvement. This method is sensitive to the nmole level and in order to measure the nucleotide of interest, tissue in gram quantities is needed which could incur sample to sample variation. The bioassay is simply not sensitive enough of a method to measure slight changes of the cyclic nucleotide. Therefore, this method significantly lacks the specificity, sensitivity and reproducibility to assay cADPR biologically.

ii. Radioimmunoassay

The second method is a more recently described radioimmunoassay for the analysis of cADPR levels in tissues (25, 51). This assay uses an antibody to cADPR that is subsequently labeled with a radioisotope and is sensitive to the picomole level. Drawbacks for the method include the observation of cross-reactivity with the anti-cADPR antiserum for the substrate β-NAD^+ and the by -product of the enzyme, ADPR and inability to purchase the antibody against cADPR, thus only a select few research groups have access to the method. With NAD, ADPR and other cross-reacting substances these being present in larger amounts than the biomolecule of interest, the sample within which the second messenger is quantitated has to be pretreated with different enzymatic digestions in order to increase the specificity of the method. Another factor

that needs to be considered is the inability to stop all cellular processes at the time of detection of cADPR therefore when measuring cADPR the nucleotide concentrations could vary. Therefore, this method is lacking the availability, specificity, sensitivity and reproducibility to assay cADPR to the levels it demands.

iii. HPLC absorbance assay

The most current, widely used method is an HPLC absorbance assay developed by Da Silva *et al.*(14). This method is reproducible and specific, yet lacks the sensitivity the detection of endogenous cADPR demands. The HPLC chromatographic absorbance assay is limited by the method of detection- absorbance. This assay lacks the sensitivity compared to our highly specific fluorescent HPLC method, which is used to quantify levels of cADPR. The current method has limits of detection sensitivity to 10 pmoles whereas our method has detection sensitivity to femtomole level. The cADPR levels were measured to be 198+41 and 28+9 pmol/10^8 cells in quiescent Jurkat and HPB ALL human T cells, respectively (26). Therefore, the cADPR levels measured were nearing the limits of their assay. Therefore, when slight changes in other systems or when minute amounts of tissue are addressed, the method will not be adequate.

iv. HPLC – Fluorescence Assay

This thesis describes the development of a fluorescent – HPLC assay that for the first time. This assay is sensitive and specific for the needs of measurement of endogenous cADPR. The method (shown in figure 4) illustrates a series of affinity chromatographic steps, enzymatic digestions, derivitization to a fluorescent form, followed by HPLC separation and fluorescence detection.

This seems to be a tedious and complex process for purification and quantification, however, the sophistication of the method is vital to achieve the reproducibility, selectivity and sensitivity that cADPR levels demand. My current studies will build upon the previous efforts of others and is described below.

1. Dihydroxyboronyl Bio-Rex 70 Resin

A physicochemical assay using affinity chromatography DHB-BioRex 70 followed by strong anion exchange high pressure chromatography in conjunction with an fluorescence detector was developed in order to measure the content of β-NAD $^{+}$ and poly ADP-ribose in intact cells. Dihydroxyboronyl Bio-Rex 70 has an ionic affinity for compounds containing 2 cis diols groups. It was previously synthesized by Wielckens *et al* (57). As previously stated, cADPR is formed after the cleavage of nicotinamide from β– NAD^{+} and therefore contains 2 cis diol groups similar to β–NAD^{+}. Thus, this resin was utilized to isolate cADPR and other related cis diol containing molecules in my assay.

2. Phosphodiesterase Treatment

Snake venom phosphodiesterase is derived from the Banded Krait *Crotalus adamanteus.* It has been shown by Kim *et al.* (30) to break down β-NAD^{+}, and ADPR, into β-NMN, and AMP; PR, and AMP, respectively, however, it leaves cADPR intact. An enzymatic digestion within the assay of the first DHB-Bio Rex 70 eluent eliminated all other cis diol containing molecules except cADPR.

3. NADase Treatment

At this point, cADPR if present in larger amounts could be quantitated. However, due to its minute levels in the cell, cADPR had to be derivatized to be measured by fluorescence. An

etheno- group cannot be added to cADPR therefore cADPR was broken down to ADPR and then derivatized.

Snake venom isolated from *Bungarus fasciatus* has NAD glycohydrolase activity (4). This venom's NADase enzymatic property was exploited in order to break cADPR into ADPR This particular NADase contains a predominant cADPR hydrolase activity that breaks cADPR into ADPR. This venom was used in the presence of EDTA to eliminate all phosphodiesterase activity that laced the venom.

4. Derivitization of ADPR to Etheno-ADPR

Following the hydrolysis conversion of cADPR to ADPR an etheno-group was added to ADPR. The derivatization process was achieved using chloroacetylaldehyde.

CHAPTER II

MATERIALS

Materials:

Cyclic adenosine diphosphoribose, adenosine monophosphate, β-nicotinamide adenine dinucleotide, nicotinamide, snake venom isolated from *Bungarus fasciatus,* and formic acid were purchased from Sigma Chemical Company, St. Louis, Missouri. Polypropylene econocolumns and BioRex 70 (200-700 mesh size) were purchased from Bio Rad, Richmond, California. M-aminophenylboronate hemisulfate, and chloroacetylaldehyde were supplied by Aldrich Chemical Company, Milwaukee, Wisconsin. The Partisil 10-SAX column was obtained from Whatman Chemical Separation Incorporation, Clifton, New Jersey. Snake venom phosphodiesterase derived from *Cratalus adamanteus* was purchased from Worthington Biochemical Corporation. Amicon Corporation, Danvers, Massachusetts provided matrix gel PBA-60. HeLa cells were purchased from ATCC. The HPLC system was purchased from Rainin and the fluorescence detector from Waters Corporation.

CHAPTER III

METHODS

Cell Culture: HeLa cells were maintained at 37° C in a humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10% fetal bovine serum.

Preparation of Dihydroxyboronyl Bio-Rex: The boronate resin was synthesized as described by Wielkens *et al* (45). Briefly, 50 g of Bio Rex-70 were placed in a 1 L flask and suspended in 600 mL of 100 mM ammonium formate buffer, pH 5.0. 5 g of m-aminophenylboronic acid hemisulfate and 5 g of N-ethyl-N'- (3-dimethyl-aminopropyl)-carbodiimide were dissolved separately in 50 mL of the same buffer and were added. The suspension was stirred very slowly with a stirring bar for 16 h at room temperature in the dark covered with parafilm and aluminum foil and the pH was adjusted to 5.0 periodically by the drop wise addition of concentrated formic acid. The resin was washed with 10 L of deionized water, with 2 L of 0.1 M ammonium formate buffer, pH 4.5, containing 1.0 M ammonium chloride followed by 2 L of 0.1 M ammonium bicarbonate buffer pH 9.0, containing 1.0 M ammonium chloride and again with 6 L of water. Finally the resin was washed with 500 mL of 500 mM ammonium formate, pH 6,0, containing 6 M Gu-HCl and stored in 200 mL of the same buffer at 4° C until used.

Affinity Chromatography Using Dihydroxyboronyl Bio-Rex 70: Affinity chromatography was carried out as described by Alvarez-Gonzalez, R., *et al.* (3). In brief, the resin was washed with 10mL of 0.25 M ammonium formate, pH 9.0. The neutralized extract was chromatographed using 10 mL of 0.25 M ammonium formate as application buffer. The resin was washed with

10mL of application buffer and eluted three times with 5 mL of 0.1 M ammonium formate, pH 4.5. The eluates were subsequently lyophlized.

Synthesis of Cyclic ADP-Ribose: A 100 uL reaction mixture containing 100 mM KH_2PO_4, pH 7.0, 500 μM β-NAD^+, 100 uCi of [^{32}P] β-NAD^+ and 0.5 units of ADP-ribosyl cyclase derived from *Aplysia californica* was incubated for 5 minutes at room temperature for the production of cADPR. The reaction was stopped at 0°C.

Purification of Cyclic ADP-Ribose: The [^{32}P] cADPR was purified by HPLC using a Partisil 10 - SAX and C18 reverse phase column placed in tandem. The reaction mixture was diluted in running buffer to 275 μL and then subjected to HPLC using an isocratic buffer system of 250 mM ammonium formate pH 4.0 at a flow rate of 1 mL / min. Fractions were collected every minute and the amount of radioactivity was determined by liquid scintillation Cerenkov counting.

Protein Determination: The amount of protein in the cell extract was determined using the BCA method (28).

Cell Harvesting: Cells were rinsed twice with 15 mL of ice-cold phosphate-buffered saline (136.9 mM NaCl- 2.7 mM KCl- 4.3 mM Na_2HPO_4- 1.5 mM KH_2PO_4, pH 7.4) and precipitated in the flask with 20% (w/v) trichloroacetic acid. The precipitated material was scraped off the flask at room temperature using a rubber policeman, and then transferred to a 50 mL polypropylene corning tube. The flask was rinsed with 20% (w/v) trichloroacetic acid again and this fraction

was pooled with the first extract. The cellular mixture was centrifuged at 3,000 rpm for 10 min and the acid soluble material was retrieved.

Phosphodiesterase Treatment: Snake venom phosphodiesterase derived from *Crotalus adamanteus* (1 unit, Worthington Biochemical Corp.) in the presence of buffer pH 9.0 (10 mM $MgCl_2$ and 0.25 M ammonium formate) was incubated overnight with the lyophilized affinity chromatographic eluent as described by Kim, H., *et al.* (30).

NADase Treatment: *Bungarus fasciatus* Banded Krait snake venom (0.5 U, Sigma Chemicals) in the presence of buffer 50 mM potassium phosphate pH 7.5 containing 5 mM EDTA, to irreversibly inactivate the phosphodiesterase, was incubated with the lyophilized affinity chromatographic eluent as described by Anderson, B., *et al.* (4).

Chloroacteylaldehyde Derivatization: The lyophilized affinity chromatographic eluent was treated with 119 mM chloroacetylaldehyde in the presence of 0.25 M ammonium formate, pH 4.5 for 4 hours at 60 °C as described by Sims, J.L. *et al.* (49)

Strong Anion Exchange High Pressure Liquid Chromatography: For the quantification of cADPR, ε-ADPR and ε-AMP nucleotides were separated from one another by HPLC using a Partisil 10 SAX column. Aliquots of each sample was routinely adjusted to pH 4.0 and diluted with deionized water and injected to the system. The solvent system utilized 50 mM KH_2PO_4 pH 4.0 buffer (low salt isocratic system) and the flow rate was 1.0 mL/ min. Absorbance was monitored at 254 nm and fluorescence was monitored by Waters system at an excitation

wavelength of 312 and emission wavelength of 425nm. The area under the peak was integrated using a Rainin system previously standardized with calibration curves of ε-AMP and ε-ADPR. Samples were placed onto a Partisil 10- SAX column with a running buffer of 50 mM KH_2PO_4, pH 4.7.

CHAPTER IV

RESULTS

I. ^{32}P cADPR PRODUCTION

a. Separation of Nucleotides by Partisil 10 SAX and C18 Reverse Phase Columns in Tandem

Figure 3 displays the chromatographic separation of standards of the reaction products and substrate of the enzyme ADP-ribosyl cyclase using the Partisil 10 - SAX in tandem with a C18 reverse phase column. Cyclic-ADPR, ADP-ribose, nicotinamide, and β-NAD^+ were separated at 10, 17, 19, and 40 min, respectively.

b. Distribution of radiolabeled and absorbance Standard ^{32}P- NAD^+ using Partisil 10 SAX and C18 Reverse Phase Columns

Figure 4A shows a chromatogram of the substrate [^{32}P] β-NAD^+, before incubation with the ADPR cyclase. This panel shows a single peak of radioactivity eluting at 40 minutes. The corresponding radioactivity (cpm) observed is exhibited with a bar graph illustrating most of the radioactivity associated with β-NAD^+.

c. Distribution of radiolabeled and absorbance Standard ^{32}P- NAD^+ after incubation with ADP-ribosyl cyclase using Partisil 10 SAX and C18 Reverse Phase Columns

Figure 4B shows the chromatogram observed after incubation of β-NAD^+ with ADP-ribosyl cyclase for 5 minutes. Incubation of the substrate with the enzyme produces a shift in absorbance (Figure 4) from the β-NAD^+ position to the elution time of cADPR. The corresponding radioactivity is also illustrated using a bar graph overlaid upon the HPLC chromatogram matching the shift in absorbance.

II. HPLC- FLUORESCENCE ASSAY

A. Maximum Binding Capacity of Dihydroxyboronyl BioRex 70 Resin for cADPR

In order to test the binding capacity of DHB-BioRex70, 10,000 cpm of ^{32}P cADPR and β-NAD^+ in increasing amounts were used. As seen in the Figure 5, up to 24 μmoles of NAD could be effectively bound to the resin. However, after this point the β-NAD^+ displaced the ^{32}P - cADPR and the curve began to plateau off.

B. Derivitization of cADPR to ε-cADPR

Treatment of cADPR with chloroacetylaldehyde did not change the retention time of cADPR on the SAX column nor was it associated with an increase in fluorescence (data not shown).

C. Calibration Curve for ε-ADPR

ε-ADPR was separated using Partisil 10-SAX HPLC followed by detection upon the fluorescence detection as shown in Figure 6. The fluorescence was determined using a fluorescence detector set at an excitation wavelength 312 nm and emission wavelength of 425 nm. ε-ADPR standards elute from the column at 29 min. A standard curve for the quantification of ε-ADPR is illustrated in Figure 6 and chromatograms associated with them illustrated in Figure 7. The Figure 6 illustrates that the peak height is directly proportional to the amount of ε-ADPR with a correlation coefficient value of 0.983. A linear relationship of the ε-ADPR was found from 1 pmol to 20 pmoles of ε-ADPR.

D. Negative Controls for the Method (Figure 8)

1. Phosphodiesterase Treatment

Without phosphodiesterase in the digestion method, the chromatogram is complex with many peaks.

2. NADase Digestion

The method run using a negative control of NADase is shown with the chromatogram to not have a peak associated with ε-ADPR (Figure 8).

3. Chloroacetylaldehyde Treatment

The method without chloroacetylaldehyde is unable to convert ADPR to ε-ADPR and therefore a peak does not have a peak associated with ε-ADPR as seen in Figure 8.

E. Positive Controls for the Method

Figure 9 illustrates HPLC analysis after phosphodiesterase, NADase (Fig. 10) and chloroacetylaldehyde (Fig. 11) treatments using known amounts of the standard nucleotides. It was found after phosphodiesterase treatment only cADPR remains intact and therefore is the only nucleotide bound to the DHB-BioRex 70 resin when analyzed by HPLC Partisil 10-SAX. After NADase treatment, the chromatogram illustrates that NADase effectively converts cADPR to ADPR. Consequently, after chloroacetylaldehyde treatment, chloroacetylaldehyde placed an etheno-group onto ADPR to give ε-ADPR detectable by fluorescence using the Partisil 10-SAX HPLC. Figure 12 demonstrates that the reagents used in this procedure do not contribute to any signal in the ε-ADPR region.

III. MEASUREMENT OF CYCLIC ADP-RIBOSE IN HELA CELLS

HeLa cell extracts spiked with ^{32}P cADPR run through the entire method gives a recovery of 40 to 50% when following the Cerenkov counts associated with the ^{32}P (Fig. 13). Also, HeLa cells spiked with ^{32}P cADPR and 25nmoles of cADPR gives about the same percentage of recovery. Of importance is that the recovery of each of the steps of chromatography is approximately 90% in order to have an overall recovery of 50%. Using the method described above, HeLa cells were found to contain 9.8 pmoles of cADPR per 10^6 cells (Fig. 14).

DISCUSSION

I. IMPORTANCE OF CYCLIC ADPR

Cyclic ADPR is a ubiquitously found cyclic nucleotide. It had been shown to be involved in Ca^{++} release that leads to CICR, fertilization, differentiation, contraction, secretion, and programmed cell death. The realm of importance of this small nucleotide is extremely broad. It is described as a conserved means of Ca^{++} release from protist to mammalian systems however, the mechanism and processes that lead up to and down from this biologically significant molecule is still a mystery.

II. ADVANTAGE OF TECHNIQUES

In brief, the method described herein measures cADPR directly, whereas the bioassay measures Ca^{++}. This is a biochemical assay that is able to measure cADPR and not only an epitope that easily cross reacts with the substrate NAD or ADPR as in the RIA. Lastly the method is simply more sensitive over the HPLC assay described by da Silva *et al.* (14).

a. Limitations of current methods

Current available methods are simply inadequate for measuring cADPR endogenously as previously stated in my introduction. The sea urchin release assay does not measure cADPR directly and instead only measures Ca^{++} and therefore it is not a reliable assay due to the other Ca^{2+} mobilizing agents present in the environment. Also, the radioimmunoassay is a method that only allows a select few to have access to because of inaccessibility commercially available antibodies. The RIA lacks specificity for cADPR and elimination is required of the substrate β–NAD^+ and the byproduct APDR to prevent cross reactivity. In addition, one cannot stop all the cellular processes in order to measure the cADPR that could possibly lead to degradation /

production of the cyclic nucleotide and thus inflated / deflated numbers could be concluded. And lastly, the third method is a cumbersome HPLC absorbance assay whereas our method is one that uses fluorescence. This gives us an increase of sensitivity of approximately 1000 fold over the previous method.

b. Importance of Fluorescent HPLC Assay

The novel assay described in this thesis is of utmost importance in this area of research. It allows for the direct measurement of cADPR in a reproducible, specific and highly sensitive manner. This method has the potential to unveil the roles of cADPR in cell systems where we did not know cADPR even existed. It is a powerful tool that measures cADPR to the femtomole level before and after illicitations with drugs, ligands, etc. This was not entirely possible before this method existed and therefore this new method represents a vital, important piece of discovery in this area of calcium mobilization research.

c. Significance of increase in sensitivity

The sensitivity of other assays is no more than 10 pmoles whereas the detection sensitivity limits of our method is to 500 femtomoles. The sensitivity of this method can therefore extend research in multiple directions. The forefront of research has almost come to a standstill due to the inability of cADPR to be measured at such minute endogenous levels.

There is usually a range within which the sensitivity of a method is optimum and beyond which limits the reproducibility of the method is compromised. The previously described methods are able to detect cADPR at the limits of their method's sensitivity and therefore the reproducibility of such methods is questionable. Without having large amounts of tissues or cells present in order to measure the cyclic nucleotide it is impossible to even detect it using the previously described methods.

d. Elimination of Lyophilization

As discussed before the AG-MP1 method (6,7) and other HPLC procedures that resolve cADPR have previously been described. However, they are less sensitive, effective and/or more time consuming. For example, a two-step HPLC method, which consists of strong anion exchange chromatography, followed by ion pair reversed phase HPLC after lyophilization of the sample recently developed by da Silva *et al.* (14). It only detects to the level of 10 pmoles of cADPR and involves a step of lyophilization. Our method eliminates the step of lyophilization by placing the strong anion exchange column in tandem with the reverse phase column to achieve separation.

III. FLUORESCENCE HPLC METHOD

I have developed a reproducible, specific and highly sensitive method for the analysis and quantification of cADPR in cellular samples. The procedure involves harvesting of cells using a 20% (w/v) trichloroacetic acid treatment to stop cellular metabolism. The TCA soluble extract was taken through a dihydroxyboronyl- Bio Rex column for affinity chromatography purification of nucleotides containing two or more riboses. Purified nucleotides were then treated with phosphodiesterase to hydrolyze all phosphoanhydride bonds. Under these conditions cADPR remains intact due to its cyclic structure. The enzymatic degradation products were isolated using dihydroxyboronyl -Bio Rex again. Next, we converted cADPR to ADPR using NAD glycohydrolase isolated from *Bungarus fasciatus* and the products generated were purified again using a third step of affinity chromatography with the dihydroxyboronyl – Bio Rex. ADPR was derivitized to the ε–ADPR fluorescent form via chloroacetylaldehyde at 60°C. The ε-ADPR was

purified on a boronate PBA-60 column and quantified by HPLC on a Partisil 10-SAX followed by fluorescence detection.

This method allows for the quantitative determination of intracellular cADPR at the picomole level directly. Our control experiments conclusively rule out the possibility of spontaneous degradation. It has been demonstrated that phosphodiesterase will functionally convert β-NAD^+ and ADPR into degradative products leaving cADPR intact (22). I have functionally shown that NADase will convert cADPR to ADPR and that the chloroacetylaldehyde treatment converts ADPR to ε-ADPR. The method shows that the HPLC successfully separates similar nucleotide containing compounds from one another and thus allowing for the direct quantification of ε-ADPR as direct products of cADPR. This method is an essential tool for the quantification of cADPR intracellularly and will allow for further insight into its signaling functions.

While this appears to be a tedious and complex process for a purification, the sophistication of the method is vital to achieve the reproducibility, selectivity, and sensitivity needed for accurate cADPR level determination.

a. Maximum Binding Capacity of Dihydroxyboronyl BioRex 70 Resin for cADPR

DHB - BioRex 70 resin has an affinity for cis-diols. It has previously been shown to bind to β–NAD^+ on a one-to-one basis (3) and cyclic ADP-ribose is a metabolite of NAD without the nicotinamide moiety and thus contains the same number of cis diol groups. Therefore, it was chosen to have β-NAD^+ as a competitor for ^{32}P cADPR to measure the binding capacity of cADPR because cyclic- ADPR is commercially an expensive reagent. The β-NAD^+ applied up to 24 μmoles effectively bound to the resin in addition to the ^{32}P cADPR, after which the curve

plateaus and β–NAD^+ begins to displace cADPR. Therefore, the maximum binding capacity of the resin can be described as 24 μmoles of cADPR.

b. Phosphodiesterase Treatment

As stated previously, isolation of cis diol containing compounds with the DHB-Bio Rex 70 was accomplished. A phosphodiesterase enzymatic digestion was vital to eliminating all cis-diol containing compounds other than cADPR. Cyclic ADPR is not digestable by phosphodiesterase and thus remains intact and still retains the ability to bind to DHB BioRex 70 resin.

c. Derivitization of cADPR to ε-cADPR

At this point in the method, detection of picomole amounts of cADPR isolated from relatively a large amount of cells was already accomplished a couple of years prior to publication by Da Silva's (12) group. However, drawbacks to an absorbance assay were understood and therefore ways to improve it to become a more powerful assay were pursued.

I immediately assumed as is the case for free ADPR measurement, cADPR could also be derivitized and measured by fluorescence. However, as was illustrated in the chromatogram it was found that this was impossible (Fig. 15). Although it was later illustrated, ε-NAD^+ can be digested with ADPR cyclase to form ε-cADPR as described by Schuber (36).

d. NADase Digestion

Turning to the enzymatic pathways, the breakdown of cADPR to ADPR is a common hydrolytic property of the NADases. This aspect of the enzyme was exploited. However, another problem arose. Cyclic ADPR hydrolase was not available in the commercial market. Either the enzyme would have to be isolated or venom which containing a predominant hydrolase would have to be utilized whereupon the other enzymes lacing the venom would have

to be inhibited. Thus the latter was done and tested with pure cADPR converted to ADPR and visualized by HPLC absorbance as seen in the figure 10.

e. Chloroacetylaldehyde Treatment

This now allowed for the simple conversion of ADPR to ε-ADPR by chloroacetylaldehyde. This procedure is one that has been done for many years to measure Poly-ADPR levels in cells by fluorescence (Figure 11).

f. Negative Controls

In order to test the efficacy of the method negative controls were performed. This was done to illustrate the importance of each of the enzymatic steps (phosphodiesterase and NADase) and that derivatization by chloroacetylaldehyde is vital for the measurement of cADPR by HPLC – fluorescence.

1. Phosphodiesterase Negative control

Without phosphodiesterase, the method is shown to have many peaks associated with the chromatogram. These were not eliminated with a phosphodiesterase digestion. These were subsequently digested with NADase and derivitized to a fluorescent form. Thus, it is illustrated that phosphodiesterase is vital to eliminating all other cis diol containing groups except for cADPR (Figure 8).

2. NADase Negative control

Without NADase, the method is ineffective in measuring cADPR at minute levels. Before digestion with NADase, cADPR has been isolated for the purpose a NADase digestion to simply break the cyclic structure. Cyclic ADPR is unable to be derivitized without the NADase breaking it first into ADPR and thus is not detected as a peak in a chromatogram as illustrated in Figure 8.

3. Chloroacetylaldehyde Negative Control

The method without chloroacetylaldehyde is unable to convert ADPR to ε-ADPR and therefore a peak was not detected in Figure 8.

g. Synthesis of Radioactive cADPR

In order to follow the recovery of cADPR through the method, a control was needed. A known amount of cADPR passed through the entire method could be viewed as sufficient. However, due to the tedious time frame of the method, a more efficient system was needed to illustrate the loss of cADPR at each step of the way. ^{32}P-labeled cADPR was chosen due to the ability to follow the many affinity chromatography steps simply by Cerenkov counting. However, ^{32}P-cADPR is not available commercially and therefore previous methods were pursued to produce labeled cADPR. However, the purification of the nucleotide was not efficient enough. There was slight overlap of cADPR with both the substrate and the by-products of the reaction. This could be detrimental to observations made regarding our recovery. Thus, a more efficient and faster method was pursued in the laboratory to produce and isolate ^{32}P-labeled- cADPR.

In other HPLC methods that separate adenine-containing nucleotides, cADPR is well resolved. However, β-NAD^+, nicotinamide, and ADP-ribose elute close to one another. Our method is more efficient because it separates the substrate, β-NAD^+ (40 min), from the product, cADPR (10 min), almost 30 minutes from each other. ADP-ribose, a potential degradation product, elutes at approximately 17 minutes right after cADPR. Also, nicotinamide, the by-product of the reaction elutes after cADPR at approximately 19 minutes. This eliminates the potential of either the nucleotides or nicotinamide overlapping with the peak of cADPR. This is of extreme importance when the radiolabeled - cADPR is used for further experimentation.

Previously, it has been demonstrated that the cyclase reaction product, nicotinamide, can inhibit calcium signaling (21). Also, the degradation product of cADPR, ADP-ribose, inhibits cADPR hydrolase activity by potentiating the production of cADPR further (21). Therefore, a need for a method to separate the products from simply the un-reacted substrate and degradation products to prevent the above scenarios is quite critical.

High pressure liquid chromatographic analysis of cADPR has also been achieved with a 3.9 x 300 mm Bondapak C18 column eluting with 100mM potassium phosphate buffer, pH 6.0 at a flow rate of 1 mL / min by Jacobson *et al.* (29). This method allows for the separation of cADPR (5 min), ADPR (6 min), nicotinamide (12 min) and β-NAD^+ (20 min) (29). However as previously discussed, due to the close retention times of cADPR (5 min) and ADPR (6 min) there is potential in this method for the cross-contamination of peaks.

Previously, Kim *et al.* in 1993 (30) described a 12.5 cm Partisil 5 SAX and a 0.46 by 12.5 cm Bondapak C18 column attached in tandem using 0.25 M ammonium formate pH 4.0 at a flow rate of 1ml/min. This method separated the nucleotides cADPR (10 min), AMP (12 min), ADPR (17 min) and β-NAD^+ (21 min) from each other. This procedure is similar to ours nevertheless due to the length of the columns the retention times of the standards is closer together, increasing the risk of cross-contamination of peaks when producing large amounts of [^{32}P] cADPR. The potential for cross contamination of peaks, also eliminates the possibility of reusing the unreacted substrate if needed.

In summary, a considerable improvement in terms of efficiency and convenience has been achieved with our new HPLC method. The application of this rapid, reproducible, sensitive, and simple method should facilitate the preparation and purification of radiolabeled cADPR synthesized from β-NAD^+ by pure ADP-ribosyl cyclase.

IV. TESTING HYPOTHESES TO DETERMINE THE MECHANISM OF cADPR ACTION IN CELLULAR FUNCTION

The importance of the novel highly sensitive fluorescent HPLC assay that I have devised and developed is vital to understanding the mechanisms of action and in testing hypotheses relating to cADPR function in living cells. This is of extreme importance due to the significance of cADPR's role in signal transduction and its ability to induce calcium induced calcium release. Without the advent of my assay and its subsequent availability to other investigators it will be nearly impossible to quantitatively assess the role of many calcium mobilizers and immunosuppressants, etc. that are dependent on cADPR function and that are important in many areas of medicine such as: cancer research, diabetes, channel blockers, and immunotherapy. The realm of importance of both the direct and regulatory function of cADPR is without limit.

Examples of the application of this method for testing important mechanistic hypothesis include the following:

A. Rapamycin

1. Introduction

The properties of rapamycin are described below as is its relationship with cADPR and a discussion of how the utilization of the above assay I have described herein can be used to test the hypothesis that cADPR plays a role in the signal transduction mechanism of rapamycin.

Rapamycin was originally described as a fungicide produced by *Streptomyces hygroscopicus* isolated from soil at Easter Island (45). Later, it was demonstrated to contain potent antimicrobial, immunosupppressant and antitumor properties (55). These properties are resultant of rapamycin's ability to modulate synthesis of specific proteins needed for cell cycle progression from the G1 to the S phase.

As an anticancer treatment strategy, rapamycin is used as a translational regulatory apparatus. It currently is in phase III clinical trials as an immunosuppressive drug for bone marrow transplant recipients. Rapamycin is hypothesized to inhibit the progression of interleukin - 2 stimulated T-cells from G1 to the S phase of the cell cycle.

Rapamycin has also been shown to augment cisplatin - induced apoptosis in murine T cells, the human promyelocytic cell line HL-60 and the human ovarian cancer cell line SKOV3. In addition, a variety of other tumor systems are prone to the antiproliferative activity of rapamycin. This evidence suggests rapamycin is a component of the signal transduction of events leading to cell death and that the efficacy of some cytotoxic agents may be enhanced by it. The known signaling pathways of rapamycin are summarized in the following figure 16.
FKBP-12 and rapamycin bind and subsequently rapamycin inhibits FKBP-12's enzymatic activity as a prolyl isomerase.

Yeast mutants lacking FKBP12.6 are viable. Yet they are resistant to rapamycin, demonstrating both the protein and drug are required for rapamycin action. The cellular effects of rapamycin are dependent upon the availibility of FKBP-12. The FKBP-12 and rapamycin complex interacts and blocks the activity of mTOR (also known as FRAP, RAFT1 and RAPT1). TOR proteins are a part of the larger family of proteins called phosphoinositide 3 kinase (PI3K)-related kinases (PIKKs). These proteins are involved in cell - cycle progression, cell cycle checkpoints DNA repair and DNA recombination. mTOR is a phosphoprotein whose phosphorylation state is regulated by PI3 kinase PKB/Akt. PI3K and Akt are proto-oncogenes and their pathways are inhibited by the tumor suppressor PTEN. The Akt pathway inhibits apoptosis and promotes cell proliferation. Downstream of mTOR are 2 separate pathways controlling translation of different mRNAs. The first is the 40S ribosomal protein S6 kinase

$p70^{S6K}$, and the eukaryotic initiation factor (e1F)- 4E-binding protein –1 (4E BP1) it also called the PHAS-1 (phosphorylated heat and acid-stable protein). These mRNA subsets are those involved in encoding components of the protein synthesis machinery itself.

a. Cyclic ADP-Ribose and Cell Cycle

Ca^{++} fluctuations have been demonstrated to be one of the important factors in the regulation of the progression of the cell cycle. In 1997, cADPR and ADP-ribosyl cyclase oscillations was directly linked to the cell cycle in *Euglena gracilis* (38). An increase in the enzyme's activity was observed in the G2 phase. Also, cyclic ADP-ribose levels were shown to be at a maximal level immediately before cell division started. The activity was measured using ^{32}P β-NAD^+ incubated with the cells that were harvested. Cyclic ADPR was measured using the previously described RIA (51).

b. Rapamycin and cADPR

There is a definite link between cADPR and rapamycin pathways – that being FKBP12. Cyclic ADPR binds to FKBP-12 subsequently releasing it from the ryanodine receptor causing it to release Ca^{++}. Rapamycin on the other hand binds to FKBP-12 in order to cause protein induced G1-S phase inhibition of the cell cycle. How is then cyclic ADPR related to rapamycin?

2. Experimental Design and Methods: I hypothesize that cyclic ADP-ribose plays a role in the signal transduction mechanism of the immunosuppressant rapamycin.

Specific Aim #1: To test the localization and functionality of CD38 and ADPR cyclase in rapamycin treated versus non-treated cells.

Specific Aim #1.1: Localization of CD38 in normal versus rapamycin treated cells.

T cells will be grown to confluency and immunohistochemical staining for CD38 and ADPR cyclase will be employed. Previously, it has been illustrated in T cells, that CD38 is located

outside of the cell and ADPR cyclase inside. Treatments with rapamycin will be compared to non-treated cells for internalization of the enzymes within these cells.

Cells will be separated into membrane and soluble portions with 100,000 g centrifugal force. Western blot analysis using antibodies against CD38 and ADPR cyclase will further illustrate localization of the enzyme to membrane versus cytosolic portions of the cell.

Specific Aim 1.2: The effect of rapamycin on ADPR cyclase activity in both CD38 and ADPR cyclase as compared to non-treated cells.

Rapamycin would be used to treat cells and the ADPR cyclase and CD38 will be isolated from. An established protocol by Graeff *et al* (24) as stated in my introduction Chapter 1, pg 9 for characterization of the enzyme will be be utilized. β-NGD^+ a fluorescent analog of NAD^+ will be used to characterize the change in functionality of the enzymes. These results will be compared to normal non-treated cells.

Anticipated results and problems

Rapamycin may or may not affect the expression of the enzymes in the cells. However, if the treatment induces a down or upregulation of the presence of cyclase within the cell and the cell transduces to induce internalization this can be seen via the techniques employed. Also, the enzyme CD38 and ADPR cyclase may not be affected by the rapamycin treatment. Therefore if the enzyme is affected to upregulate/ down regulate the production of cADPR in the cell due to the immunosuppressant this can be observed. A potential pitfall to this part of the study is that the enzyme activity remains the same and is unaffected by rapamycin. This is in itself reportable due to the lack of knowledge in this field of the enzyme.

Specific Aim #2: To determine the levels of cADPR in control and rapamycin treated cells.

The cells would be treated with and without rapamycin as in specific aim number 1. The cells would be rinsed with PBS 2X to wash excess off and then my protocol measuring cADPR via HPLC fluorescence would be employed using treated and non-treated cells.

Anticipated results and pitfalls

The results could be anticipated as one of three potential outcomes: cADPR levels go down and/ or stay the same. There is nothing described in the literature to illustrate that cADPR levels change after rapamycin treatment. However, FK506 (which also acts via the FKBP12.6) in pancreatic β cells has been described to reduce the release of Ca^{++} via the cADPR mechanism. Thus, any findings in regards to rapamycin treatment upon the cADPR levels would be reportable.

Specific Aim #3: Effect of rapamycin upon FKB12.6

Cells will be grown and immunohistochemical staining for FKBP12.6 will be used. These studies will be compared to rapamycin treated cells.

Alternative methods and pitfalls

If the enzyme does not change in localization and neither does the activity, and the cADPR levels do not change, then another variable left in the equation is the binding protein. Therefore, if localization does not change, I can look at the protein itself. Is the protein being produced in larger amounts? This can be tested by western blotting for FKBP12.6 with and without rapamycin using antibodies against FKBP12.6. And then quantified using a known amount of FKBP12.6 run in a lane in the gel as a control.

Experimental design

Can soluble FKBP12.6 bind to rapamycin? This is the first question that needs to be answered to delineate the signal transduction of events that leads to G1-S phase interruption by rapamycin.

In order to answer this question, rapamycin could be simply bound to an affinity column. FKBP12.6 next could be run through the column and vice versa. The columns would be eluted and characterized for binding. Also, FKB126 and rapamycin could be incubated together and compared to FKBP12.6 and rapamycin run separately. A shift in the molecular weight could establish an interaction between the two.

In order to further establish a link between FKBP12.6, rapamycin, and cADPR a set of experiments need to be done. The first would be to establish that these three players are in interaction with each other. To do this I would utilize ^{32}P cADPR and FKBP12.6 and rapamycin. A supershift gel assay would be run. The first lane would contain ^{32}P cADPR and FKBP12.6 incubated together and run in a gel. Because we do know these bind, by autoradiography I would see a shadow representative of this. Next, I would add rapamycin to the incubation, after ^{32}P cADPR and FKBP12.6 was allowed to bind. If FKBP12.6 complexed with cADPR interacted with rapamycin, this would cause a supershift with a label associated with it. However if rapamycin simply replaces cADPR, the supershift still would be present. However the autoradiograph would not have a signal associated with it. Controls I would include in the experiment are: rapamycin and FKBP12.6, FKBP12.6 and cADPR, FKBP12.6, rapamycin and cADPR by themselves. Potential pitfalls to this experiment include that FKBP12.6 does not bind to rapamycin when complexed with cADPR. FKBP12.6 is bound to the ryanodine receptor and it is also found to be soluble within the cell.

Finally, affinity for FKBP12.6 for cADPR could change with treatment. Therefore to illustrate this a monoclonal antibody would be chosen that does not contain an epitope that would interfere with cADPR binding. This antibody would then pick out FKB12.6 bound and un- bound to cADPR. The cADPR could then be quantified and compared to treated cells. The

anticipated results for this experiment include an upregulation of FKBP12.6 and cADPR or downregulation of these or simply no change at all in binding affinity for cADPR.

The molecular mechanism linking rapamycin and cADPR is lacking in today's research. This is of extreme clinical importance. If cADPR is connected to the signal transduction of events then perhaps other more potent agents that would inhibit cADPR binding to FKBP12.6 could be used to produce the same effects. The link to Rapamycin could simply be made by measuring cADPR levels before and after the subject to the drug.

B. B-cell chronic lymphocytic leukemia

1. Introduction

B-CLL is the most common leukemia in Western society. Approximately 7,500 people develop the disease and nearly 5000 die from it yearly. It occurs more commonly in the male population, with increasing incidence in ages above 40. It is a heterogenous disease, where some survive without much therapy and others die rapidly despite an aggressive treatment.

In order to characterize these 2 major subsets with in the disease, systems have been developed to address the prediction of patient survival. Parameters such as lymphocytic doubling time, cytogenetic abnormalities, and soluble CD 23 are being used to stage currently.

B-CLL is characterized by an accumulation of CD5+ B- cells. These are approximately 50% virgin lymphocytes and the other half previously triggered, postgerminal center memory B cells.

Greater than 30% CD38 B cell expression has been evaluated to be an accurate predictor of a worse clinical outcome in B-CLL than those patients with less 30% $CD38^+$ expression.

The physiological significance of CD38 expression in cell survival and cell proliferation is unknown. My method could take this prognostic marker of $CD38^+$ B cells and see if the

presence of the NAD glycohydrolase is of significance to the cyclic nucleotide cADPR. Thus, I hypothesize along the lines of Okamoto's model in pancreatic beta cells and insulin secretion. If cADPR levels increase, the substrate for PARP decreases and does not allow for the cell to undergo apoptosis and instead keeps living. This is the case in B-CLL, which is a disease of accumulation because apoptosis does not occur. Cyclic ADP-ribose levels could be measured in patients with greater than $CD38^{+}$ B^{-} cell expression and a poor clinical prognosis. I would predict that the levels in these cells to be higher. I hypothesize that there is a correlation of more enzyme to the presence of more nucleotide, and in patients with 30% and lower expression CD38, levels of cADPR should be much lower. Our simple assay could provide great insights to a current marker for the disease, i.e. it might be a cause of the signal transduction events leading to disease.

Experimental Design

Specific Aim #1: Levels of cADPR in diseased versus normal state.

Again this is a perfect example of an application of my method for which the question could not be answered in the absence of the sensitivity of the assay described herein. There is an increase in the amount of enzyme present in the diseased state. Does this correlate to an increased/decreased level of cADPR or is this a method by the cell to upregulate the production of cADPR due to the abnormal activity of the enzyme? In order to understand the signal transduction mechanism of B-CLL, one needs to be able to measure cADPR in a sensitive and reproducible manner.

Potential Pitfalls

A potential pitfall to this study is that cADPR levels could vary significantly from sample to sample, i.e. from lower and higher levels of cADPR and CD38 ratios. This however, is of

significance a reportable observation because studies to correlate enzyme studies could also be made.

Localization of CD38

Due to the high amount of CD38 present on the outside of the cell, one could ask the question, why is it there? Is this because CD38 is acting as a differentiation marker only? In this case the enzyme amount, activity, and localization would not change. Another scenario is that the system was compensating for lower amounts of cyclic nucleotide and more enzyme was being produced. Thus the enzymes activity would be reduced. This could be checked after isolation of the enzyme and NGD^+ (the fluorescent analog of NAD in activity testing). Next, along the lines of Le Chatelier's principle more product cADPR is being used up and therefore more is being produced. This could be easily tested with my fluorescent HPLC technique to measure the levels with in the cells in addition to observing the activity of the enzyme.

It is also of interest to determine whether CD38 is being internalized to compensate for lower levels of cyclase. This could be immunohistochemically looked at with antibodies against both the cyclase and CD38. Quantification could be assessed via western blotting for both again.

Pitfalls

Again, in this scenario, assessment for the binding protein needs to be addressed. If cADPR levels are in higher it will be of importance to ascertain the reasons for higher production. Possible reasons might include compensation for low affinity by the binding protein or no presence of the binding protein at all. The latter could be demonstrated via immunohistochemical staining to illustrate the presence of a soluble receptor.

Other pitfalls to the study might include cADPR levels might be opposite to my prediction. The enzyme is up-regulated because cADPR is found in more minute levels than

when the differentiation marker is less than 30%. However, this would nevertheless give insight to the disease events. Another pitfall might be that cADPR levels do not change, but only the differentiation marker levels do. This is just another example of where the presence of the enzyme might have nothing to do with the product. However, this also would tell us about the role of the differentiation marker and the progression of disease and lack of connection to the signal transduction of events leading to a change in cADPR.

V. SYNOPSIS

As discussed above, it is clear that this assay as discussed above suggests the huge potential application for investigation of cADPR's involvement in the arena of several biochemical pathways including its involvement in rapamycin's ability to immunosuppress and with the enzyme CD38, a now marker for CLL.

VI. CONCLUSIONS

Within my thesis studies, I have successfully developed an HPLC fluorescent assay for the measurement of cADPR. This method is of pivotal importance to the field of cADPR. The fluorescent HPLC assay is biochemically based. While this method is complex and involved the sensitivity for detecting cADPR levels found within the cells warrants the employment of this sophisticated and this state of the art technology. Without the advent of this assay, critical hypotheses, as illustrated in the discussion of this thesis, would not be possible or would be very much more difficult to test.

CHAPTER V

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Production of Cyclic ADP-Ribose

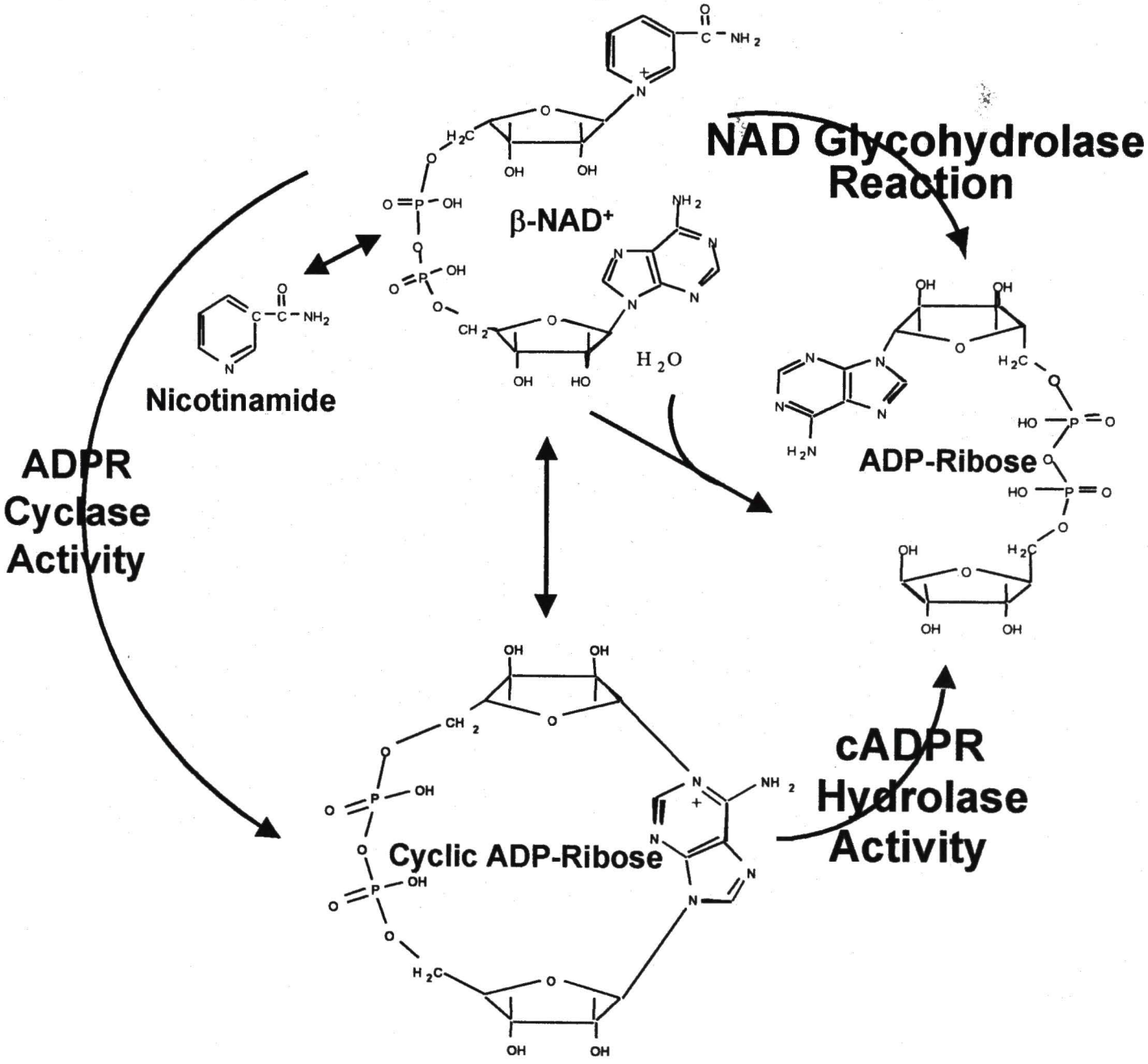


Figure 2

Distribution of Calcium Pools

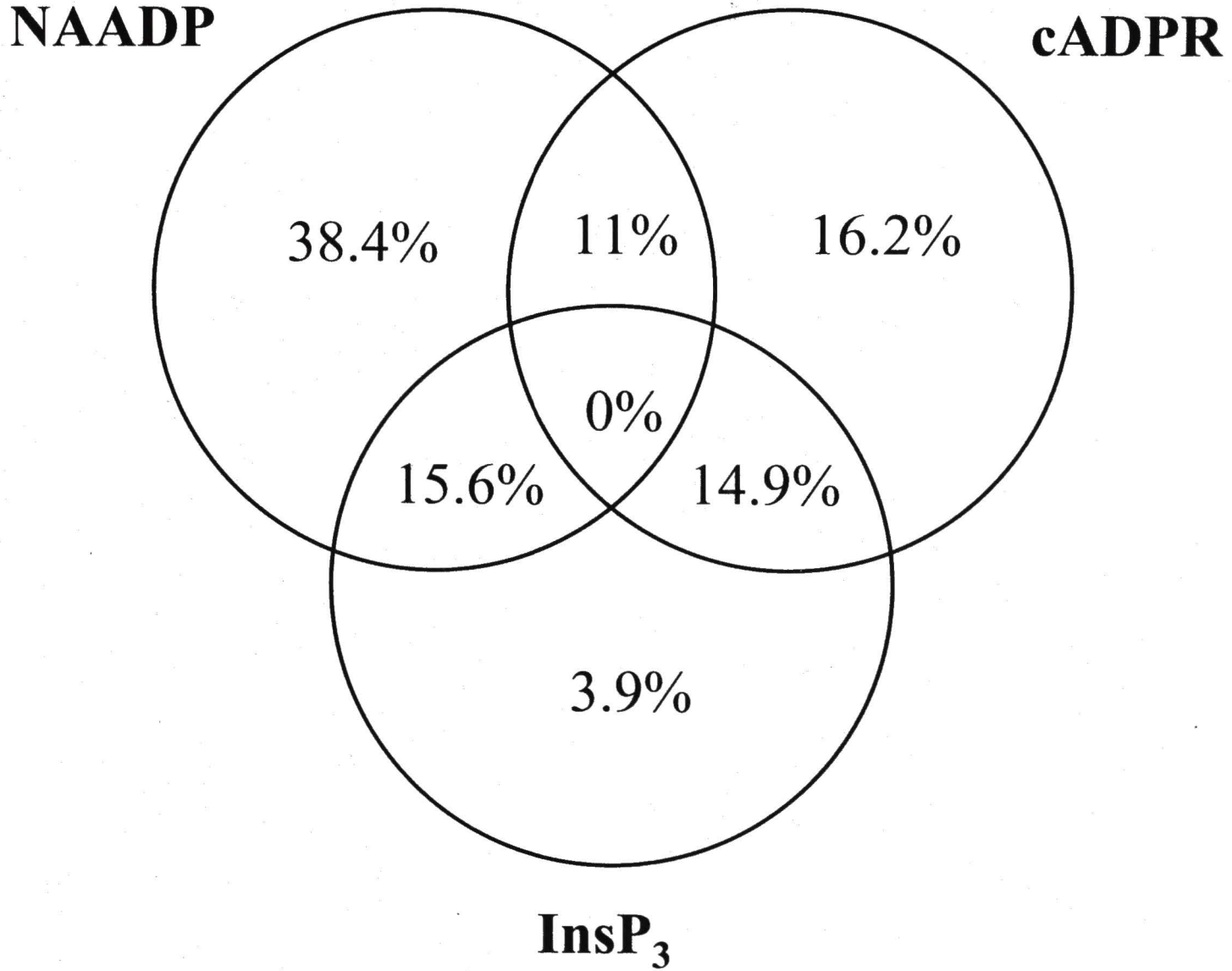
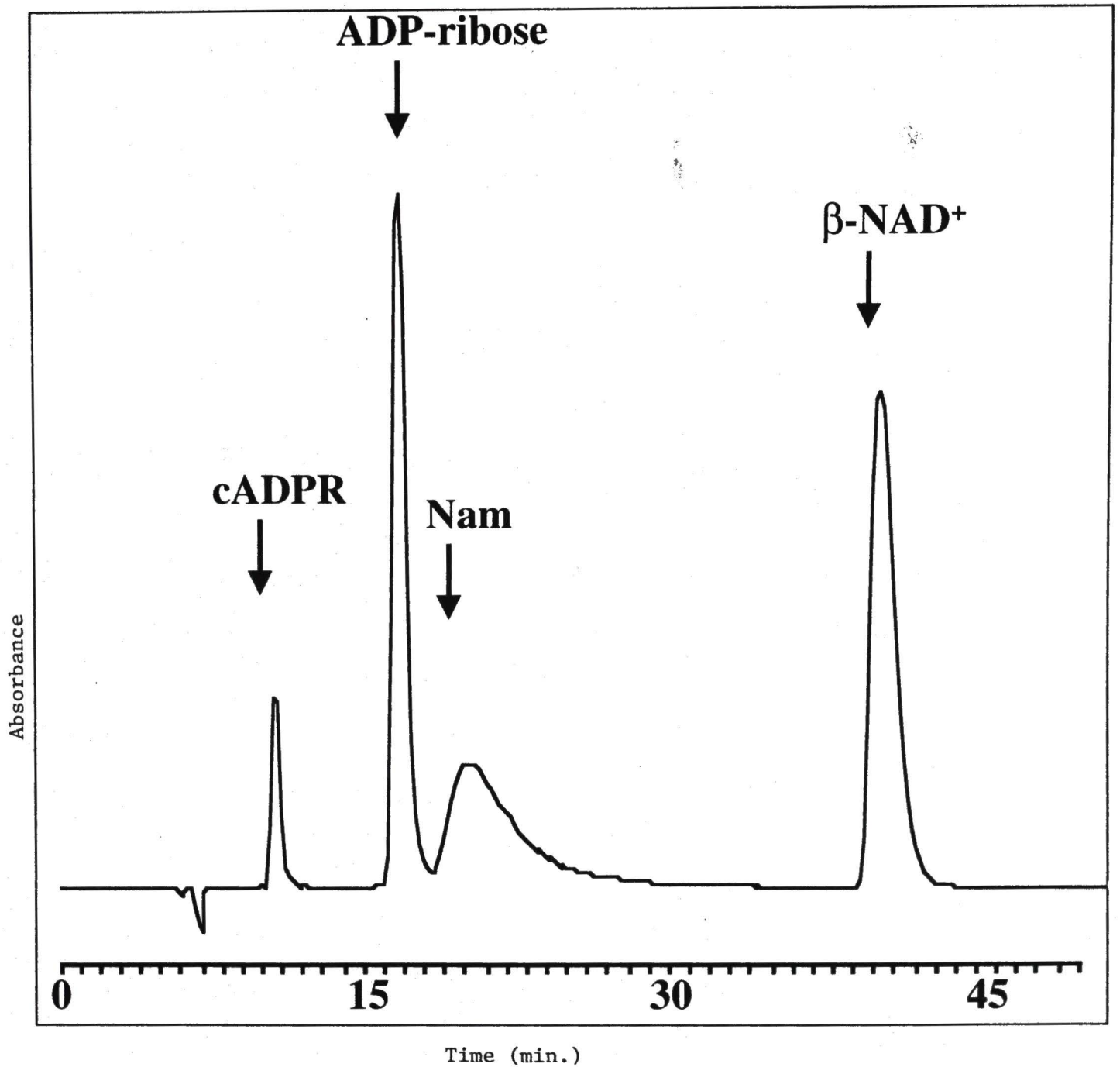
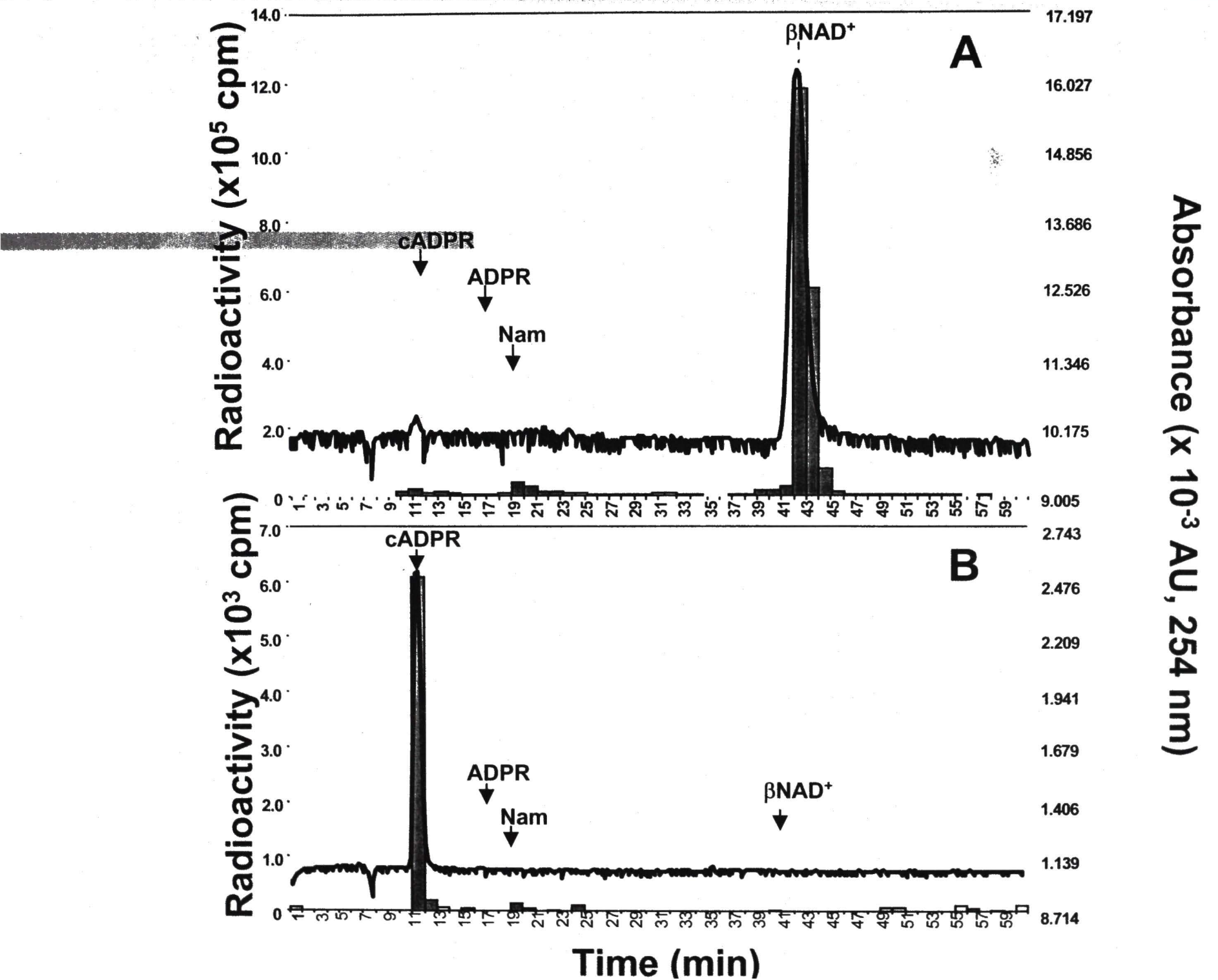


Figure 3




A
B
βNAD+
cADPR
ADPR
Nam
Radioactivity (x10⁵ cpm)
Radioactivity (x10³ cpm)
Absorbance (x 10⁻³ AU, 254 nm)
Time (min)

Maximum Binding Capacity of Dihydroxyboronyl Bio-Rex for cADPR: A Competition Assay Using NAD as a Competitor

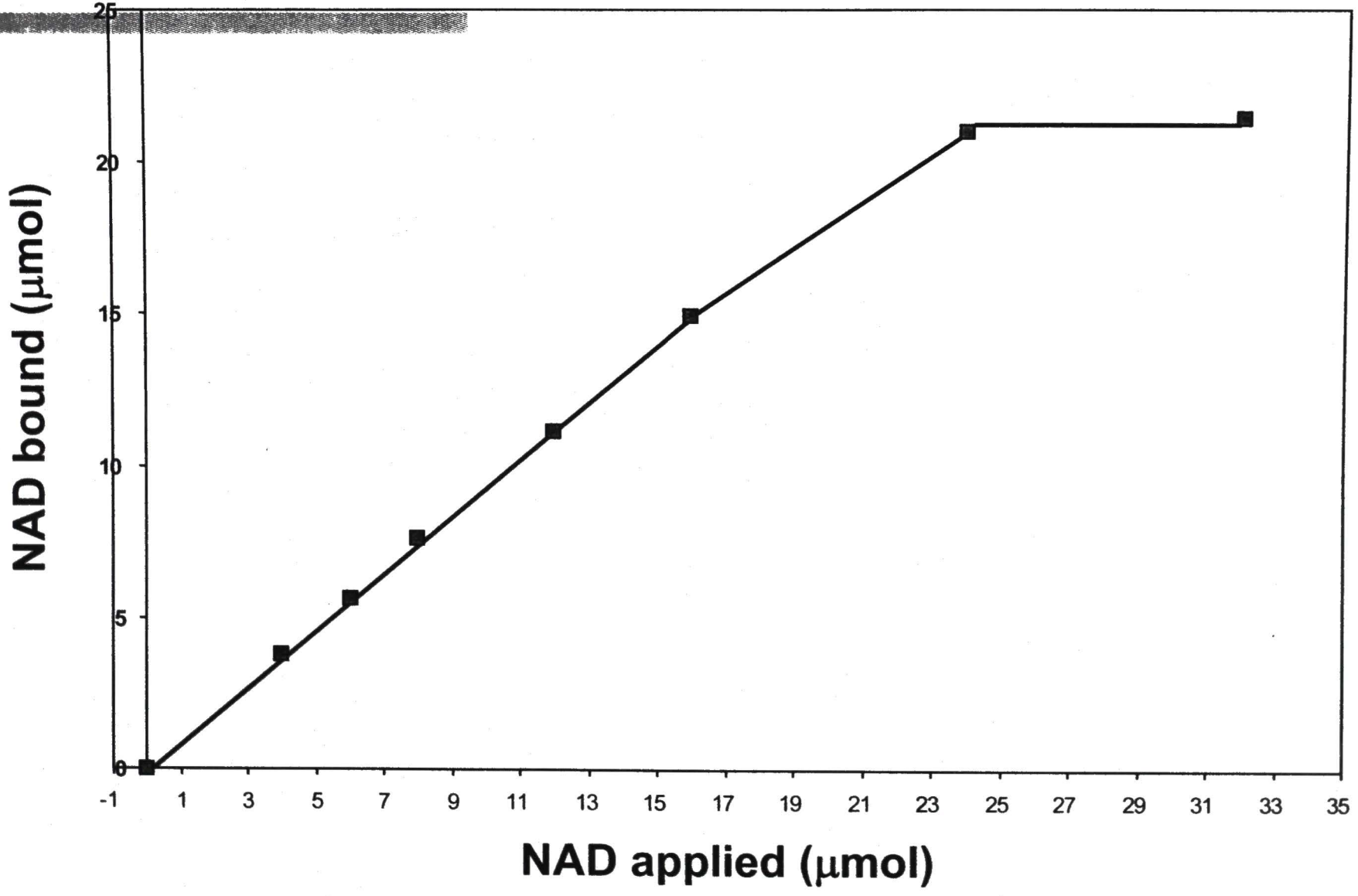


Figure 6

Standard Curve for ε-ADPR

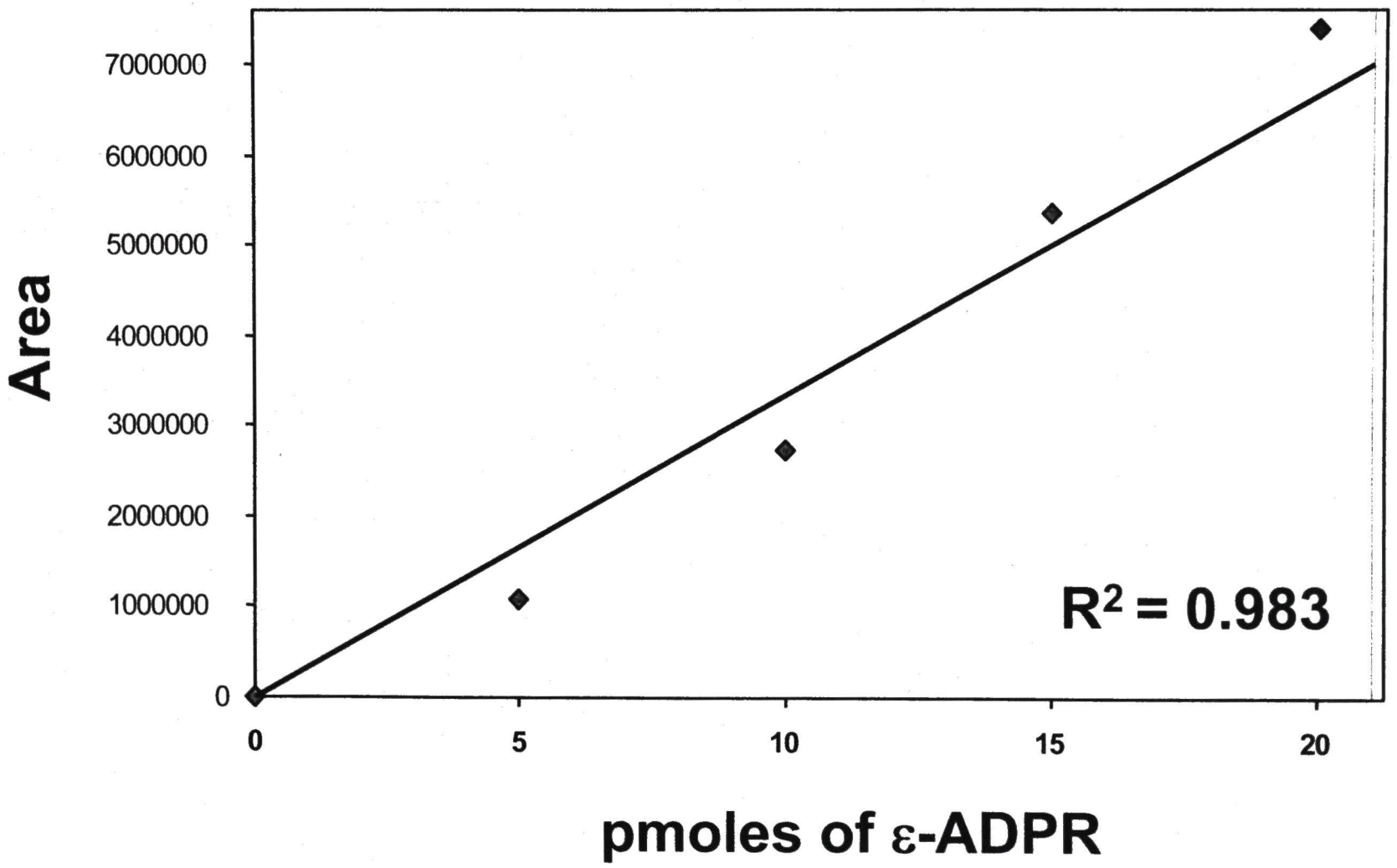


Figure 7

Chromatograms for ε-ADPR curve

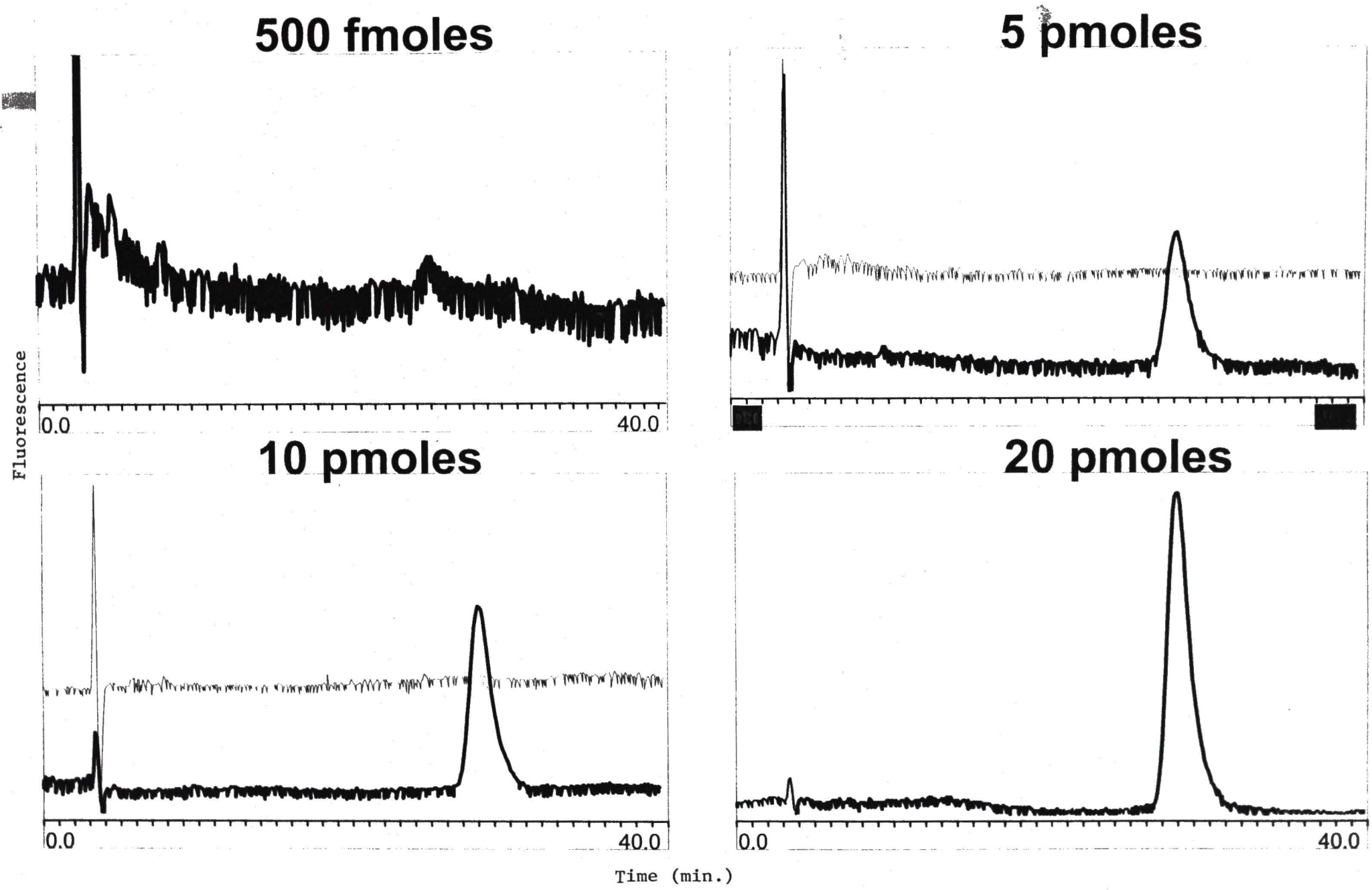


Figure 8

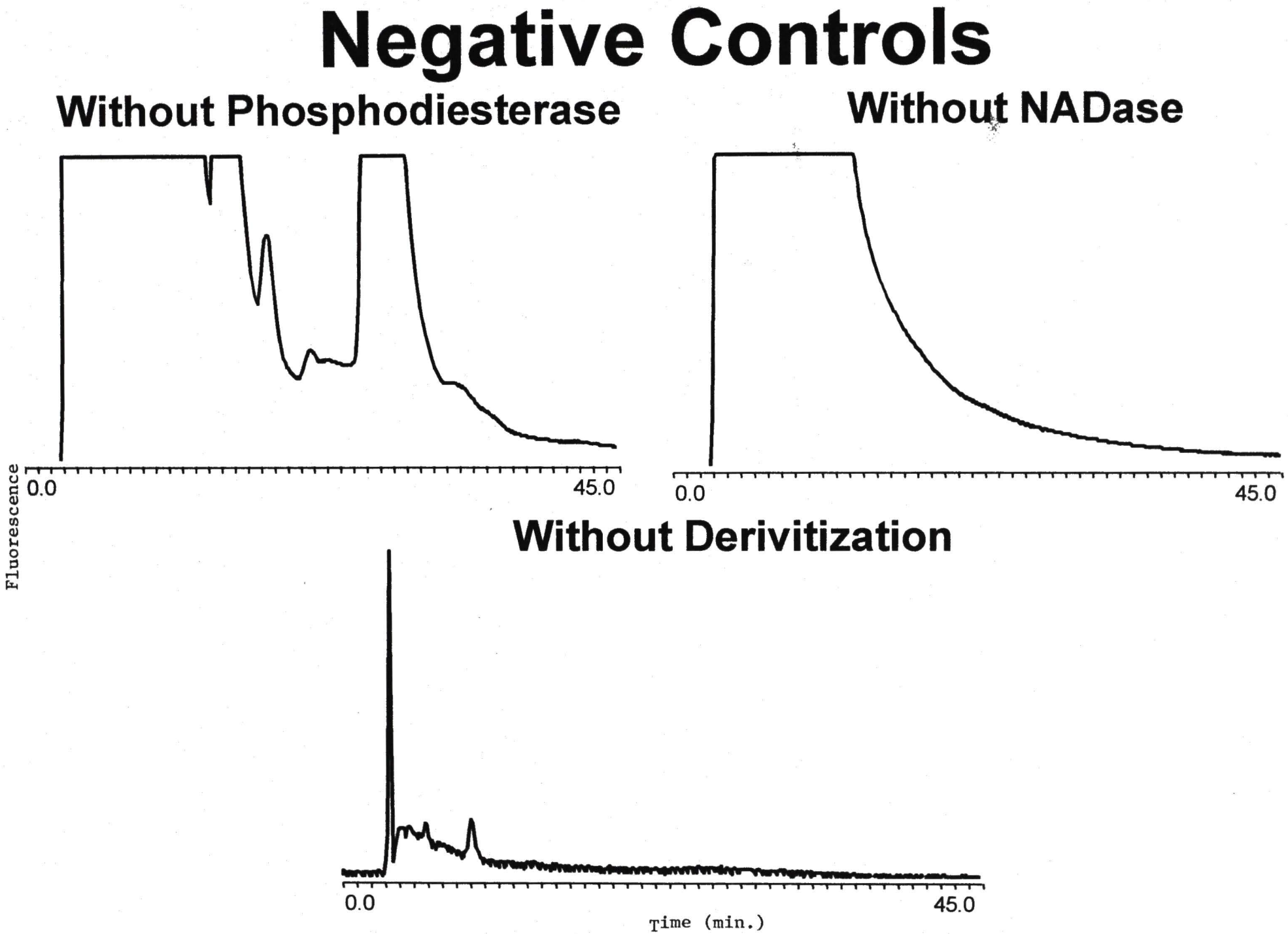

Negative Controls
Without Phosphodiesterase
Without NADase
Without Derivitization
Fluorescence
0.0
45.0
0.0
45.0
0.0
45.0
Time (min.)

Figure 5

HPLC Analysis after Phosphodiesterase, NADase, & Chloroacetylaldehyde Treatments

After Phosphodiesterase Treatment

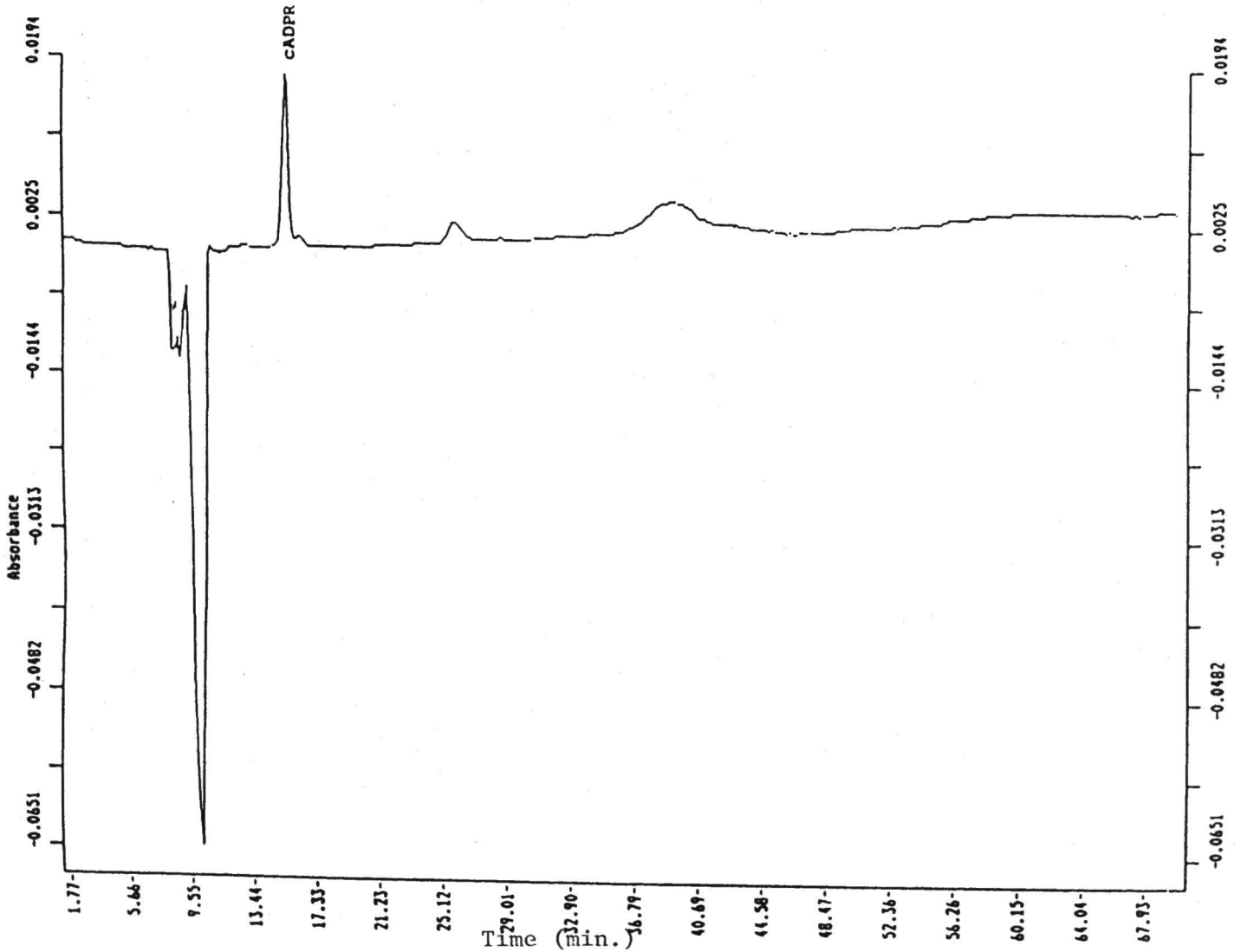
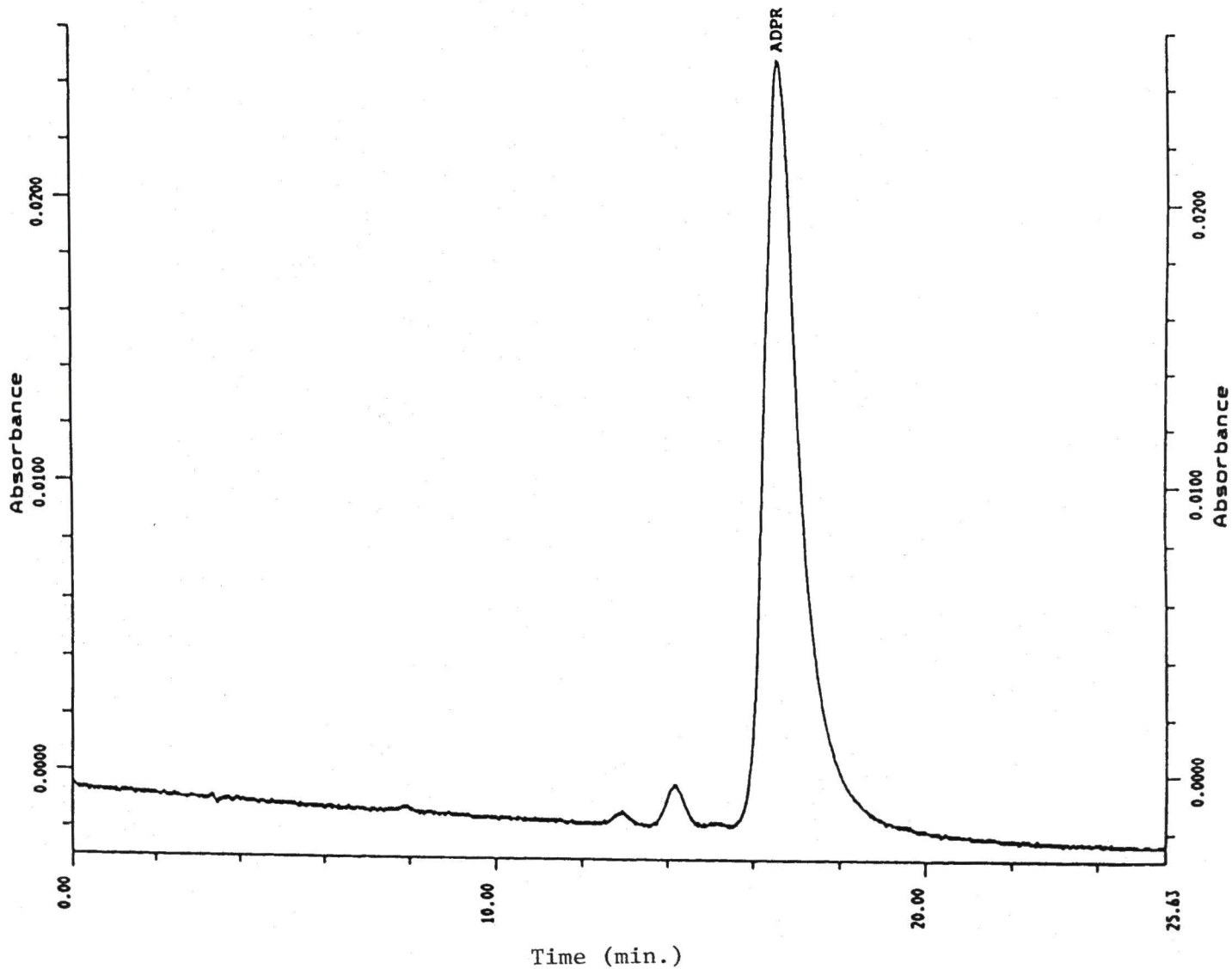
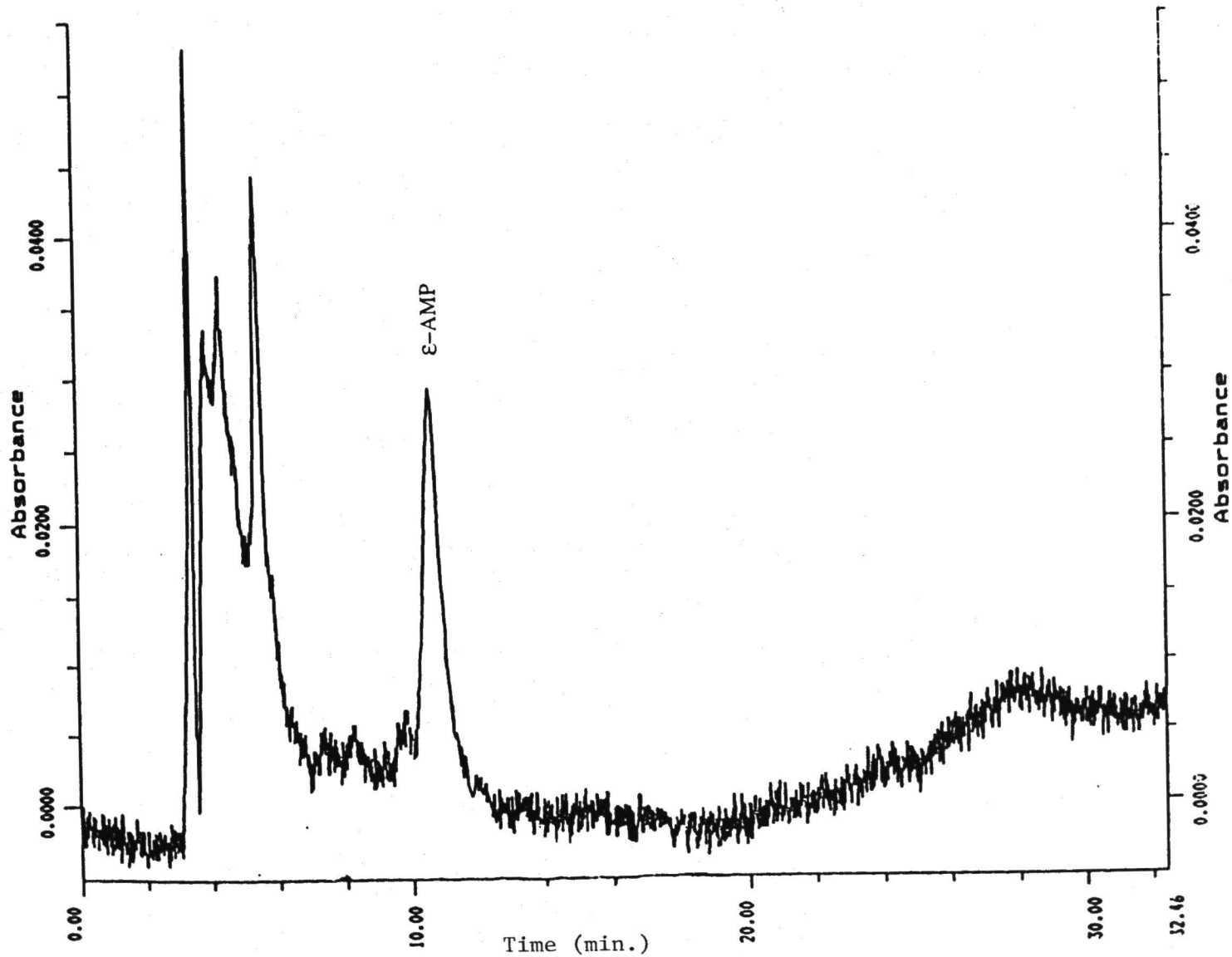


Figure 15

After NADase Treatment



After Chloroacetylaldehyde Treatment



Negative Control

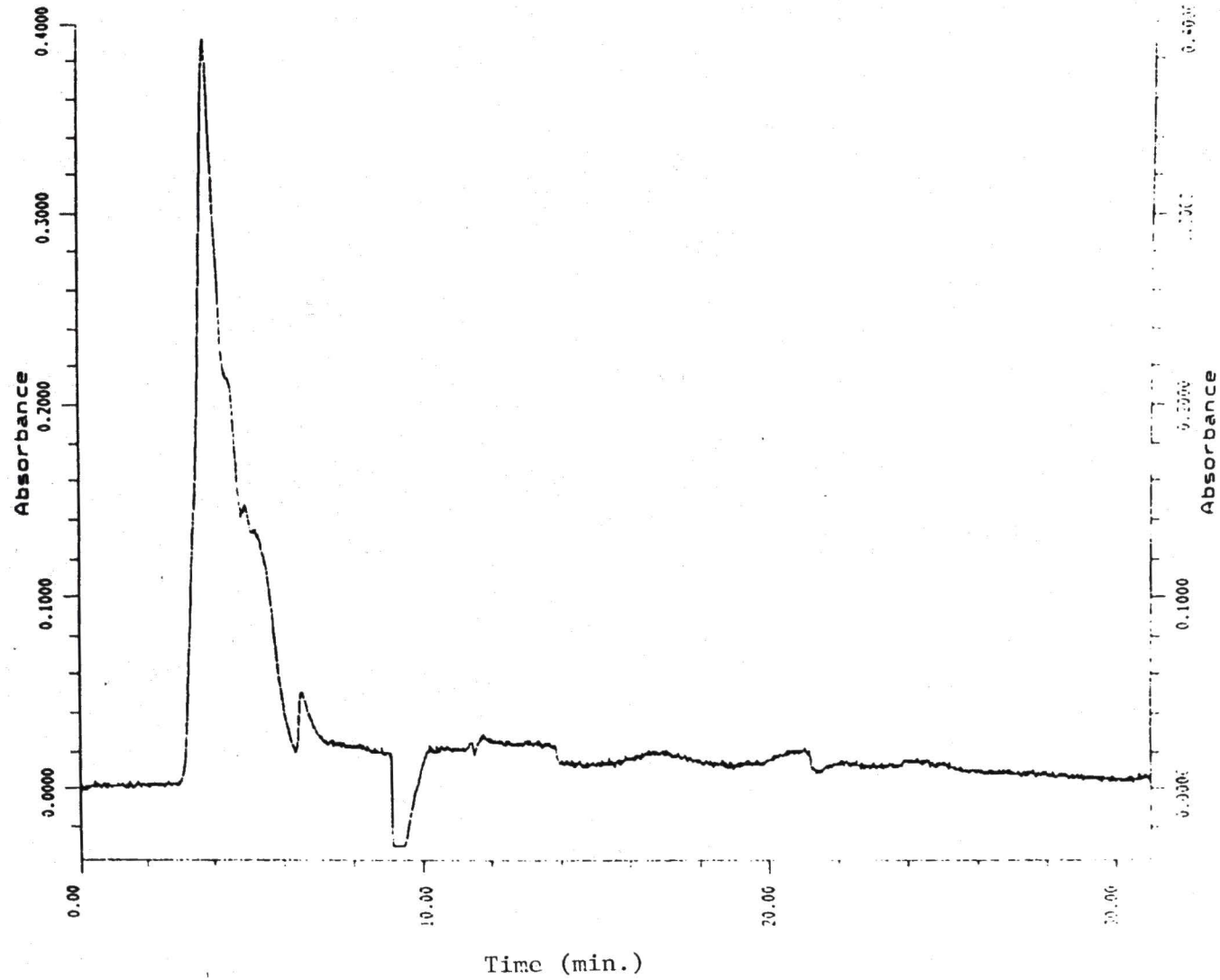


Figure 13

Recovery Controls

HeLa cells spiked with [^{32}P]- cADPR	43.3% 41.2%
HeLa cells spiked with [^{32}P]- cADPR and 25nmoles of cADPR	49.2% 53.2%

Figure 14

Experimental Results

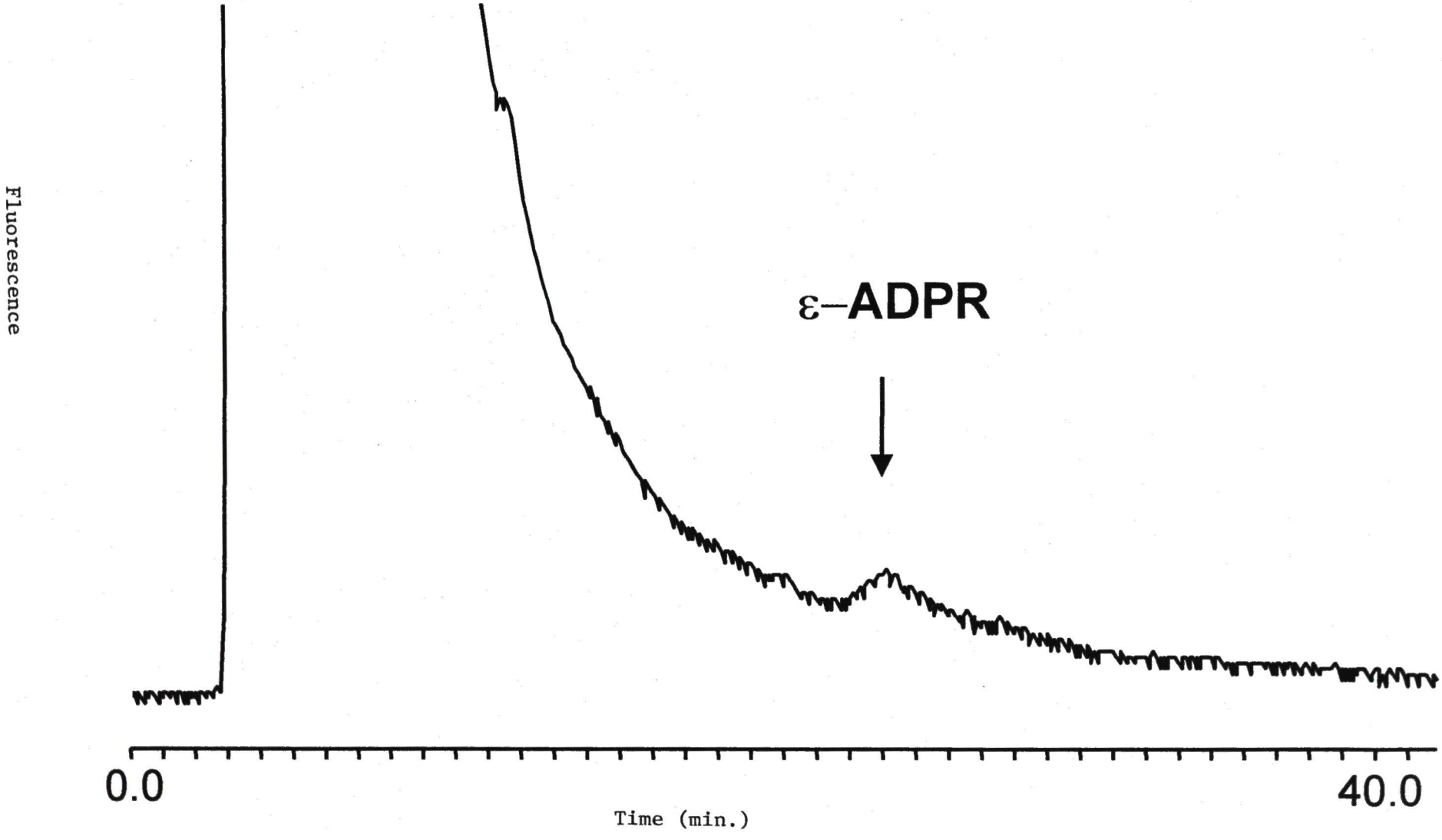
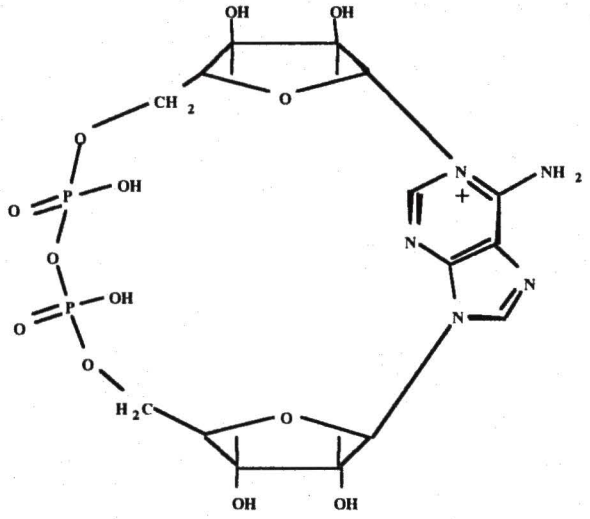
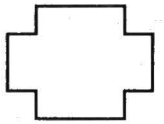


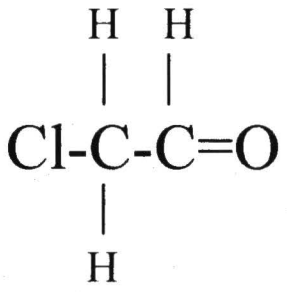
Figure 15

Production of ε-cADPR from cADPR by Chloroacetylaldehyde

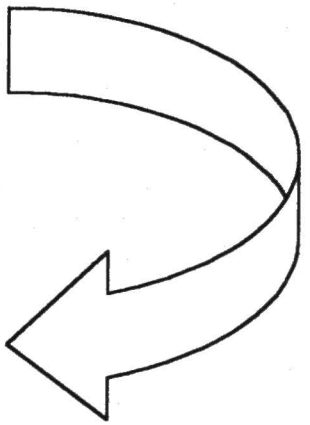


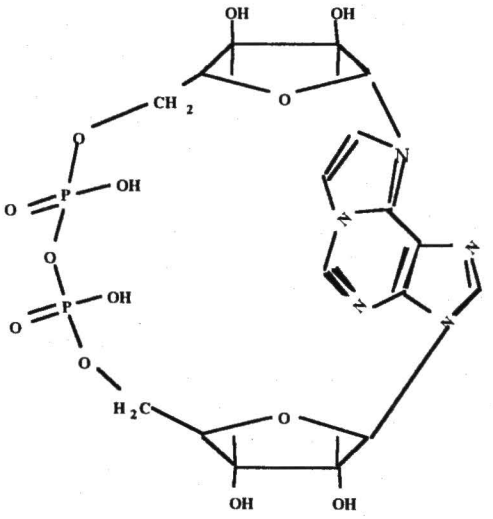
Cyclic ADP-ribose





Chloroacetylaldehyde



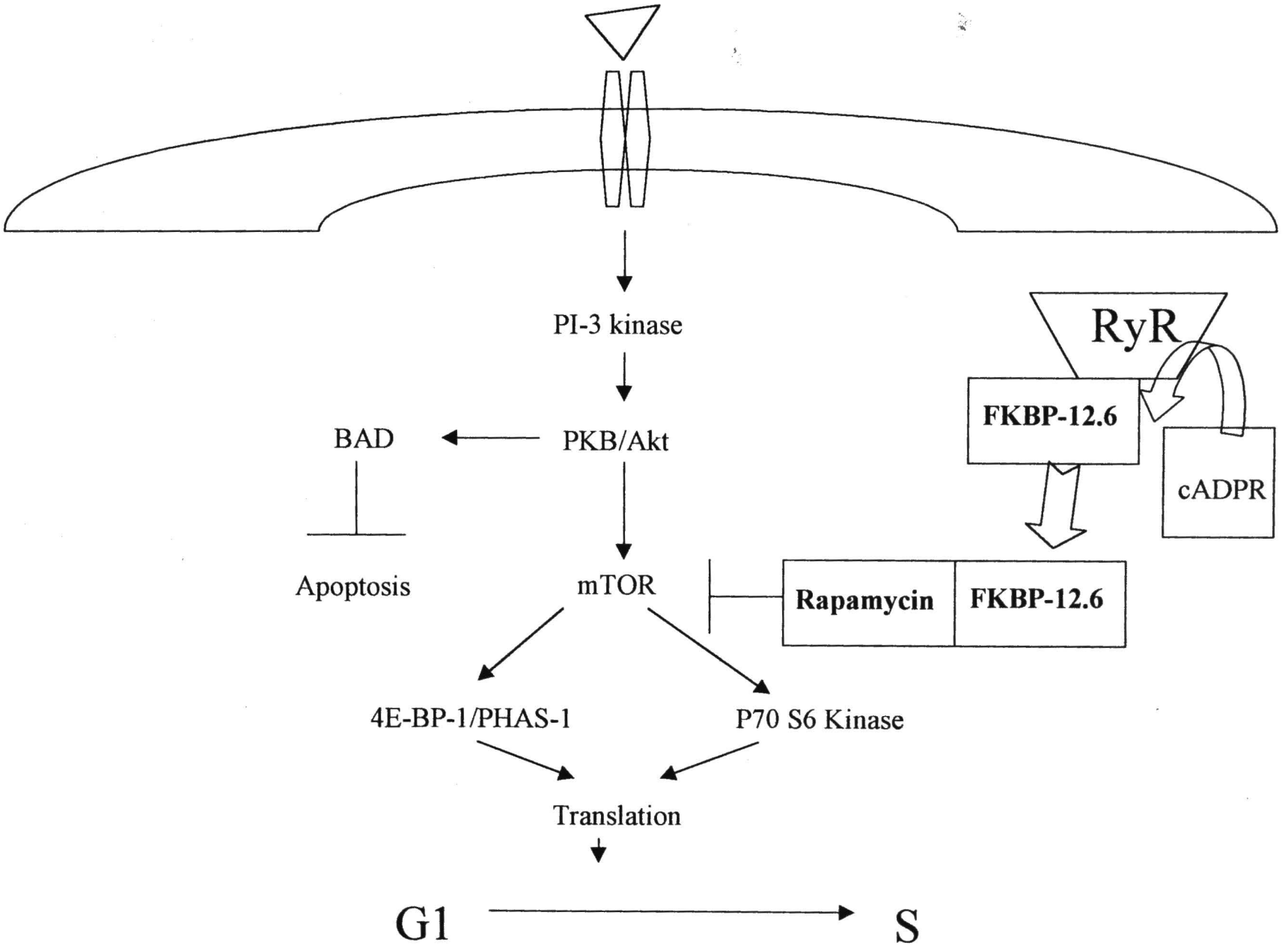


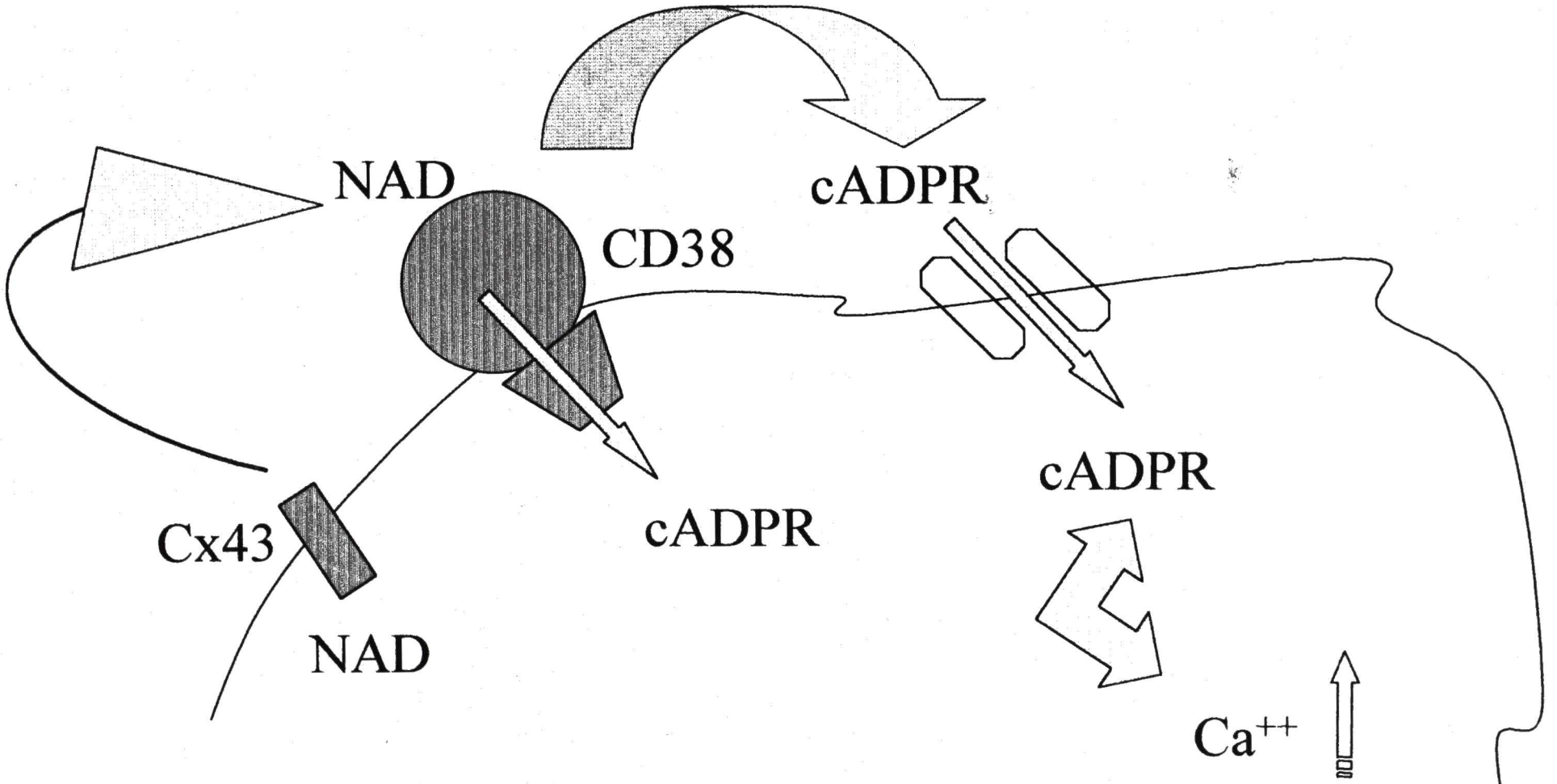
ε-cADPR

Analyze by HPLC

Figure 16

Rapamycin-Sensitive Signaling Pathways





Paracrine Roles of NAD+ and Cyclic ADP-Ribose in Increasing Intracellular Calcium and Enhancing Cell Proliferation of 3T3 Fibroblasts

By Luisa Franco, Elena Zocchi, Cesare Usai, Lucrezia Guida, Santina Bruzzone, Aurora Costa and Antonio De Flora

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