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In a study of the effects of treatment with the anti-P.C. acting drug Bryostatin-1, the levels of protein kinase C isoforms in patient peripheral blood lymphocytes was studied. While the levels of many of the isoforms was quite variable, protein kinase C eta was frequently elevated shortly after the start of treatment and then subsequently suppressed during long-term exposure to the agent. The level of MMP-9 in the plasma of these patients was highly variable and no active enzyme was detected. Studies are under way to determine if this variation is seen in subjects that are cancer free. We have generated preliminary data that suggest that PARP cleavage, Bcl-2 protein levels and Raf and MAPK phosphorylation state may be useful markers of Bryostatin action.

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FOREWORD

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Introduction

Metastasis is a complex, multi-step process that requires the coordinated expression of a number of properties by the tumor cells, which allow them to overcome the significant obstacles that lie between the primary tumor and the final metastatic site (1). These properties include altered cell-cell and cell-substratum adhesion, increased motility, the elaboration of proteases, altered growth control, and the ability to produce angiogenic factors. The mechanisms by which each of these processes are regulated are likely to be different. However, it is probable that all are modulated to some extent by the central signaling pathways used by cells. Indeed, many of the properties listed above have, for example, been shown in various model systems to be regulated by Protein Kinase-C (PKC)-mediated pathways; agents that modulate PKC have been shown to alter the rate of metastasis in some animal models. Furthermore, transfection of poorly invasive, non-metastatic cells with the PKC-α gene increased their metastatic potential.

There is an increasing body of evidence that different isoforms of PKC (a family of at least 11), are responsible for the regulation of different processes, and it was in the light of this that we originally hypothesize that metastatic behavior may in part result from: 1) the overexpression of specific isoforms of protein kinase C leading to increased activation of PKC responsive metastatic processes, and/or 2) an increase in the sensitivity of the cell to the signals that activate protein kinase C, resulting in increased kinase activity and stimulation of metastatic processes.

We have realigned the emphasis of this proposal (DAMD 17-94-4166) to take a more detailed look at the utility and effects of agents that alter PKC activity in the clinical setting. We have studied the effects of the anti PKC active agent Bryostatin-1 on the levels of the various PKC isoforms *in vivo* as part of a clinical trial of this agent. We will study what impact these changes have on the level of signaling in cells exposed to this compound and examine the induction of cell death. We will conduct a similar but more directed study in which the level of a single isoform of PKC (alpha) will be targeted using antisence therapy. These studies will be done using tumor biopsies collected before and during therapy and so will provide a unique opportunity to study the detailed effects of such modulation. We have continued to refine the tools that we are using to study the impact of the extra-cellular environment on the level of PKC activity in breast cancer cells. We have developed a unique molecular approach that will allow us to obtain detailed information about the actual level of PKC activity in various contexts.

PROGRESS REPORT FOR DAMD 17-94-J-4166

Body:

This report details the progress made towards completing the technical objectives described in the amendment to those objectives that is attached as appendix A. As directed, we are addressing the new objectives although at this time the approval for the change of emphasis of the proposal has not been granted.

Task 1. Effect of Bryostatin-1 on Protein Kinase C (PKC) isoform levels and activity *in vivo*.

A) Determination of PKC isoform levels in peripheral blood lymphocytes

Bryostatin-1 is a macrocyclic lactone that is believed to mediate the biological responses that it produces by its actions on PKC. The literature is full of conflicting data regarding the activity of this drug, and it has become increasingly clear that this is because the agent can act as a PKC activator, partial agonist or complete inhibitor. Which activity is seen depends on the system that is used, the concentration of the drug and the isoform of PKC that predominates in that system. PKC is a multi gene family coding for at least 10 separate proteins. Studies conducted *in vitro* have shown that treatment with Bryostatin-1 can result in the loss of specific isoforms of PKC from cells. This is thought to be caused by a transitory activation of these PKC molecules followed by rapid clearance of the proteins from the cell. These effects vary from system to system and depend on the concentration of drug used. Many of these studies have also been done with extremely high concentrations of Bryostatin-1 that are probably un-achievable *in vivo*. No detailed study of the effects of treatment with Bryostatin-1 on PKC levels *in vivo* has been done and so as part of an ongoing clinical trial at the cancer center, we have set out to determine if Bryostatin-1 therapy causes alterations in the level of PKC levels. We decided to study these effects in the peripheral blood lymphocytes (PBLs) for several reasons. Firstly, since we wanted to be able to study multiple time points in each patient, it was impractical to try and do the work on tumor biopsies. Second, PBLs express high levels of most of the isoforms of PKC and so the most data about the effects of the different isoforms could be generated with this tissue. Thirdly, the drug was being delivered intra-venously and so the blood cells would be the first tissue to be exposed to the drug, and interpretation of the data would not be confounded by differences in the access of the drug to the tissue being assayed. Furthermore, samples of plasma were to be kept to be assayed for the drug, and so more direct dose-response relationships could be developed in this tissue.

Thus far, the samples from 22 patients have been collected and are in various stages of evaluation for PKC levels. Briefly, blood samples are drawn into a vacuum blood tube containing EDTA as an anticoagulant. After dilution with an equal volume of PBS the blood is layered onto Ficol Hypaque gradients and spun in a centrifuge. The plasma layer is removed and the PBLs aspirated and washed with PBS. A whole cell lysate is prepared from the cells by the addition of boiling lysis buffer (10 mM Tris pH 8, 1% SDS). The samples are boiled, sonicated and a small sample is removed to determine the protein concentration. The remainder of the sample is mixed

1:1 with sample buffer and frozen until required at -70C.

PKC levels in the samples are determined by western blot analysis. Samples (20 μg) are run on 7% poly-acrylamide gels and transferred electrophoretically to nitrocellulose. After blocking of the non-specific binding sites on the membrane in 3% BSA in TBST (Tris buffered saline, 0.1% Tween-20), the blots are incubated with antibodies against the various isoforms of PKC (see below). Extensive washing is followed by detection of areas of immunoreaction with a horse radish peroxidase conjugated secondary antibody, followed by treatment with a chemiluminescent substrate for HRP and exposure to x-ray film. The level of PKC isoforms in the samples is then assessed by densitometry of the x-ray film.

Table 1 contains a summary of all which of the 10 isoforms (α, β, γ, δ, ϵ, ζ, η, θ, ι/λ and μ) that we are studying have been analyzed in each of the patients. All of these data are presented in graphical form in the pages following that. As can be seen from the graphs presented, there is a considerable amount of variation seen in the levels of various isoforms and it is difficult, with one exception to determine a strong pattern in these data. We believe that this is the result of subtle changes in the numbers of the various cell linages that are represented in the PBLs that we isolate. We know that these cells contain different levels of the various isoforms. We are in the process of developing a FACS based assay that will allow us to look at various isoform levels in specific cell linages to help address this issue.

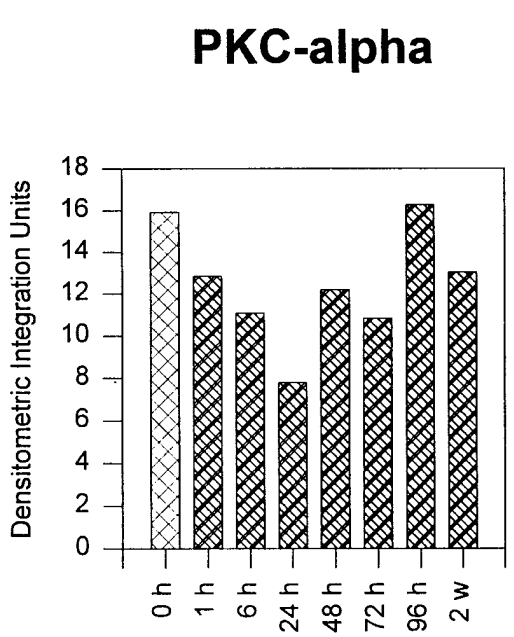
Anther possibility for the variation seen is that despite the continuous infusion of drug that these patients are receiving, there may be variations in the level of the compound in their blood. This is complicated further by the fact that it is not known what the biological half-life of the compound is under these conditions. Work is ongoing to develop an assay for Bryostatin-1 that is sensitive enough to allow a full pharmacokinetics study to be done. A plasma sample will be assayed for each time point that we have for the PKC assays and so we will be able to express the data as a function of the drug concentration. It is anticipated that this assay will be available within the next few months. It is, however, clear form the data that PKC-eta is consistently suppressed over a long period of exposure. Similarly, in most patients, there appears to be a transitory peak in PKC-eta levels. A summary of some of the PKC-eta data are presented in Fig A. We are in the process of trying to determine the cause and implications of this effect.

Over the next few months we will be continuing to analyze samples from the existing patients to fill in the gaps in the data set, and as patients are accrued to the trial we will continue to process and analyze these new samples. As soon as data about the plasma levels of the compound are available we will re-analyze our findings to determine if there are any more consistent patterns of modulation produced by Bryostaint-1. Work is also proceeding to determine which cells express the most PKC-eta, to investigate the possibility that a particular cell type is being deleted from the patients blood on long-term treatment with Bryostatin-1. We will continue to try and develop a FACS based assay for some of the PKC isoforms as this will allow us to determine the changes produced in specific cell lineages by gating the data based on the expression of cell surface markers using multi-color FACS. We hope that this will allow us to investigate more subtle shifts in PKC levels.

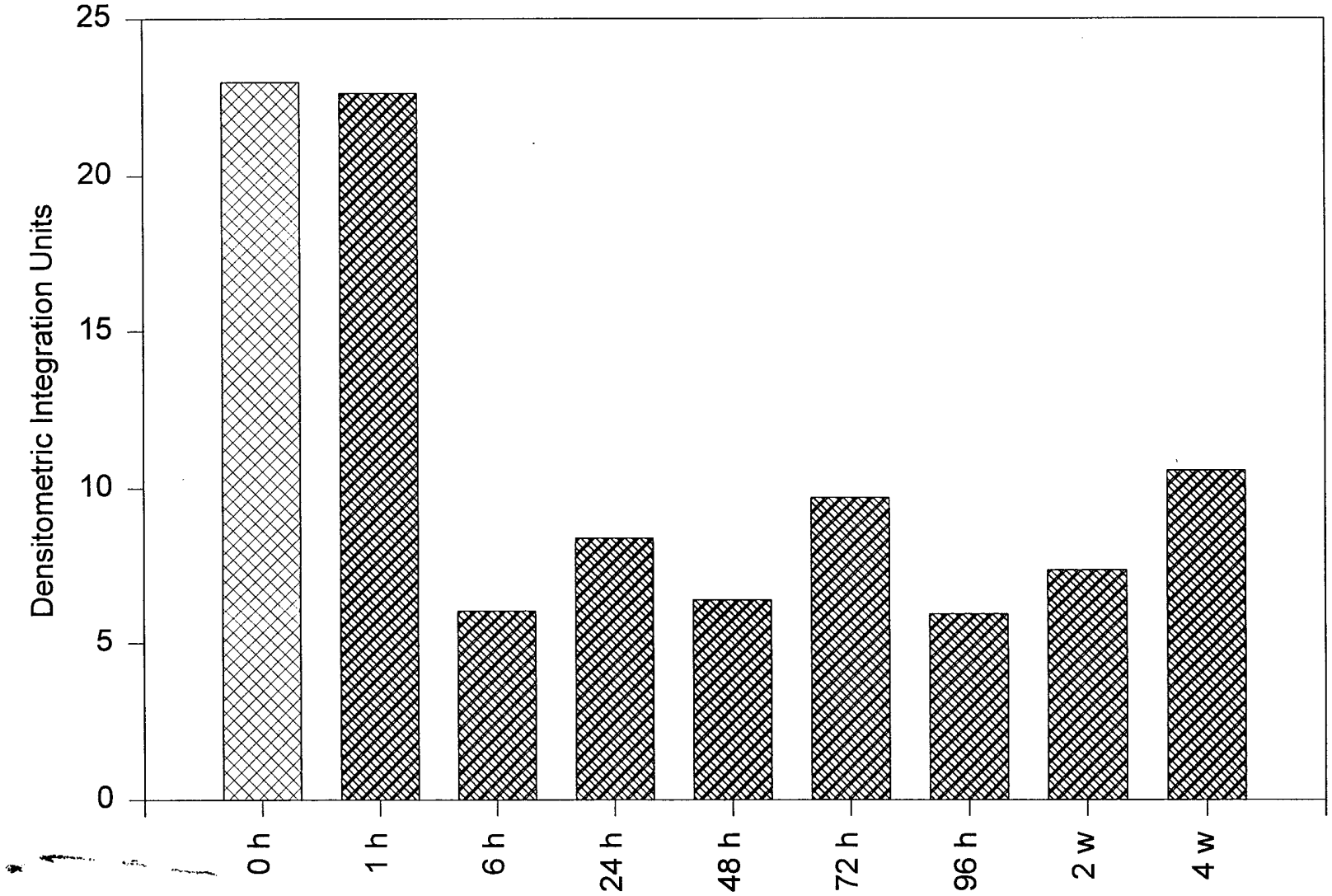
Table 1

Patient Number	MMP	alpha	beta	gamma	delta	epsilon	eta	theta	zeta	lambda	mu
1	X	X									
2	X	X		X			X				
3	X						X				
4	X				X	X	X				
5	X										
6	X				X	X	X				X
7	X			X	X	X	X				X
8	X	X		X	X	X	X			X	X
9	X	X	X	X	X	X	X		X	X	X
10	X	X		X	X	X	X			X	X
11	X	X									
12	X	X		X		X	X				
13	X										
15	X	X		X			X				
16	X	X					X				
17	X	X			X	X	X				
18	X	X			X	X	X				
19	X	X			X	X	X				
20											
21	X										
22	X	X			X		X				
23		X					X				
24		X			X		X				

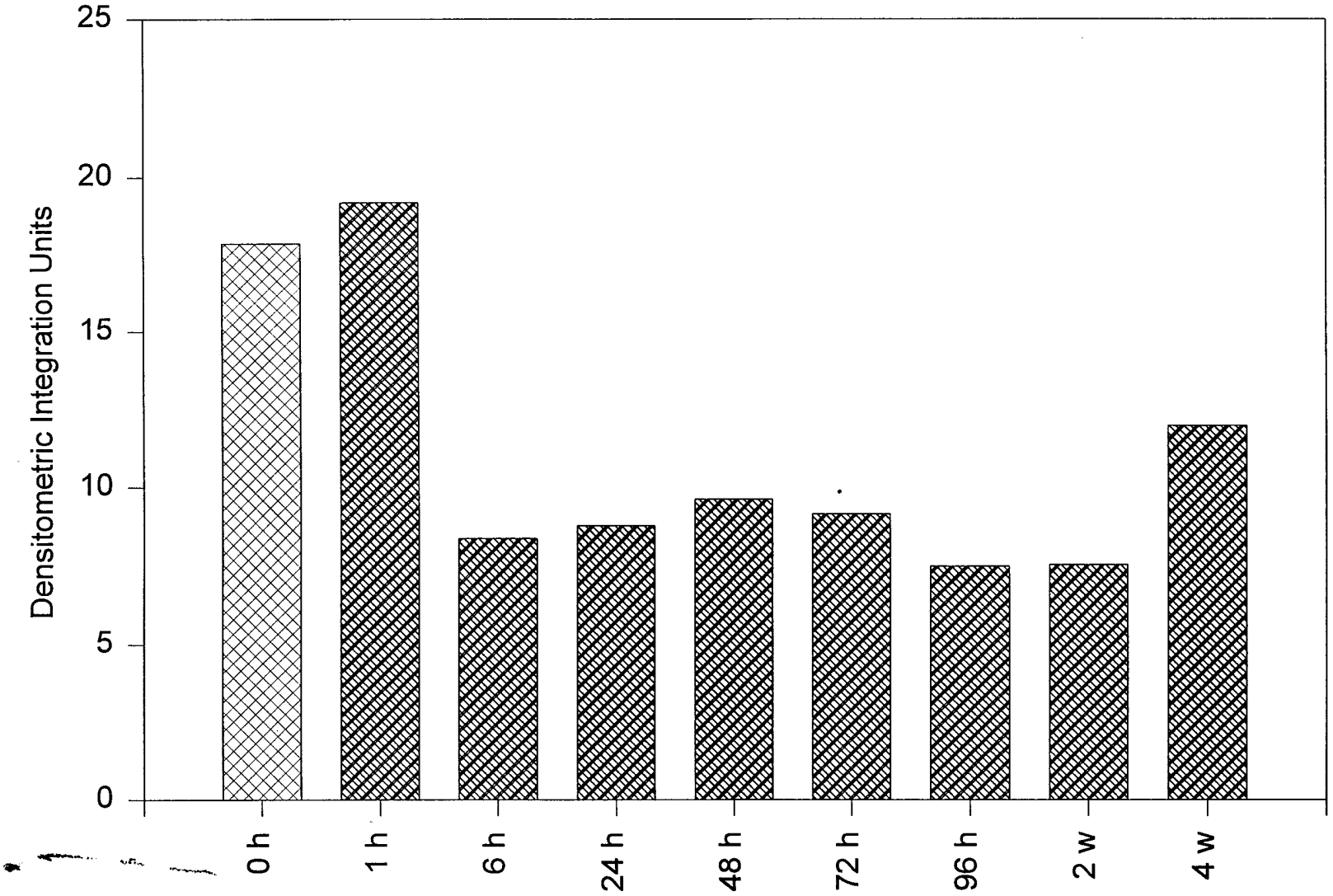
Patient #2: Protein Kinase C Levels vs. Treatment with Bryostatin-1



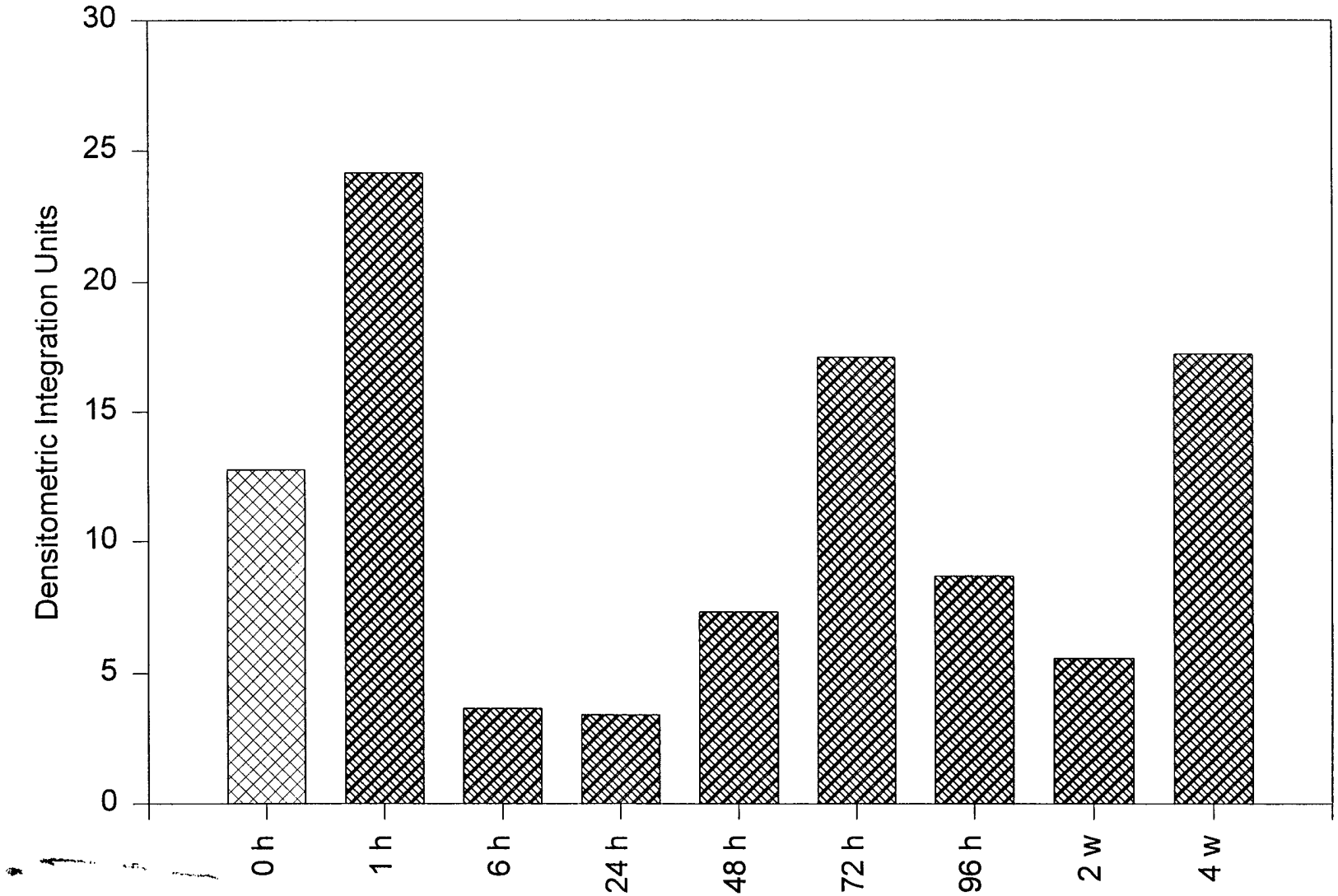
PKC-delta Levels in Patient #4 vs. Treatment with Bryostatin-1



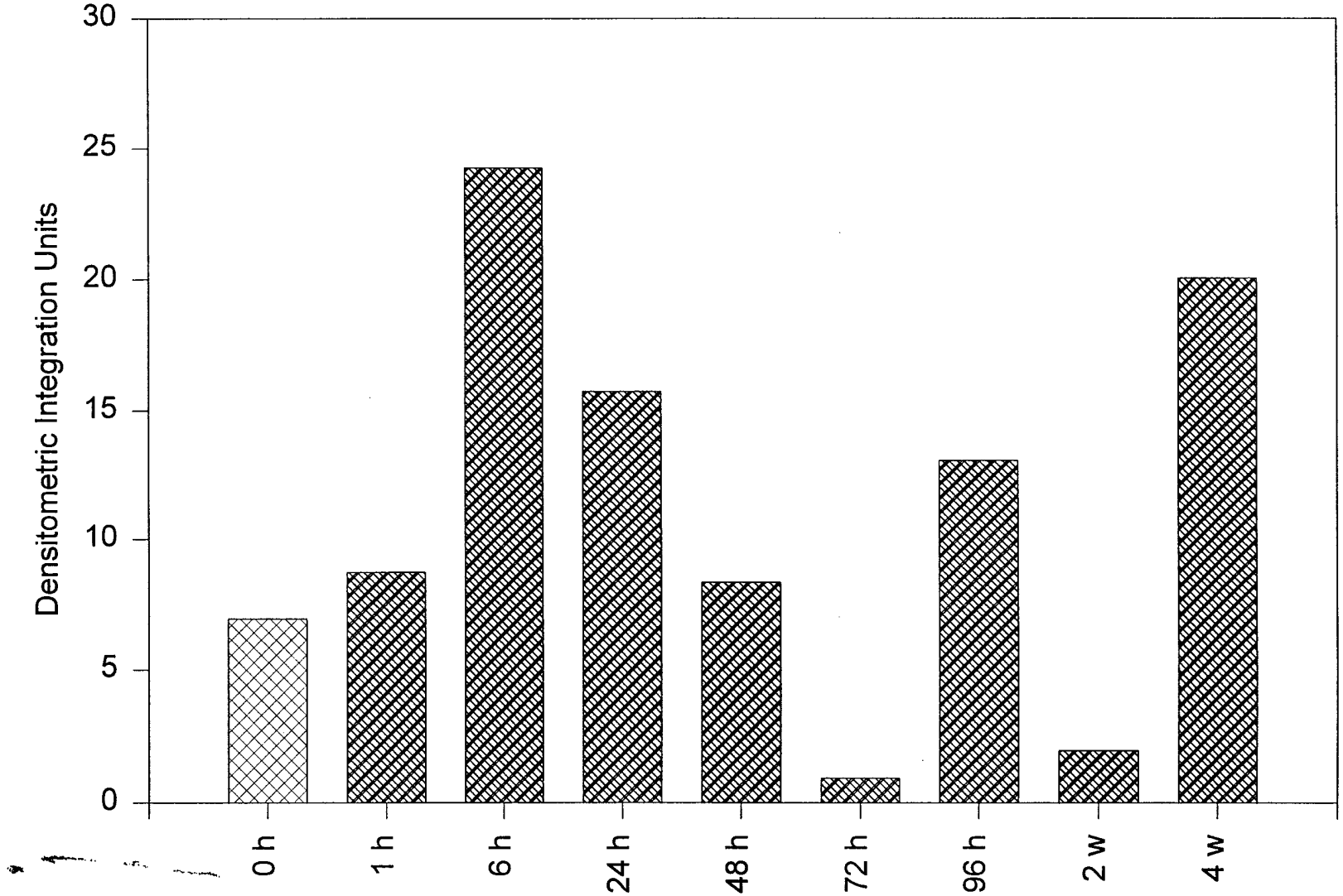
PKC-epsilon Levels in Patient #4 vs. Treatment with Bryostatin-1



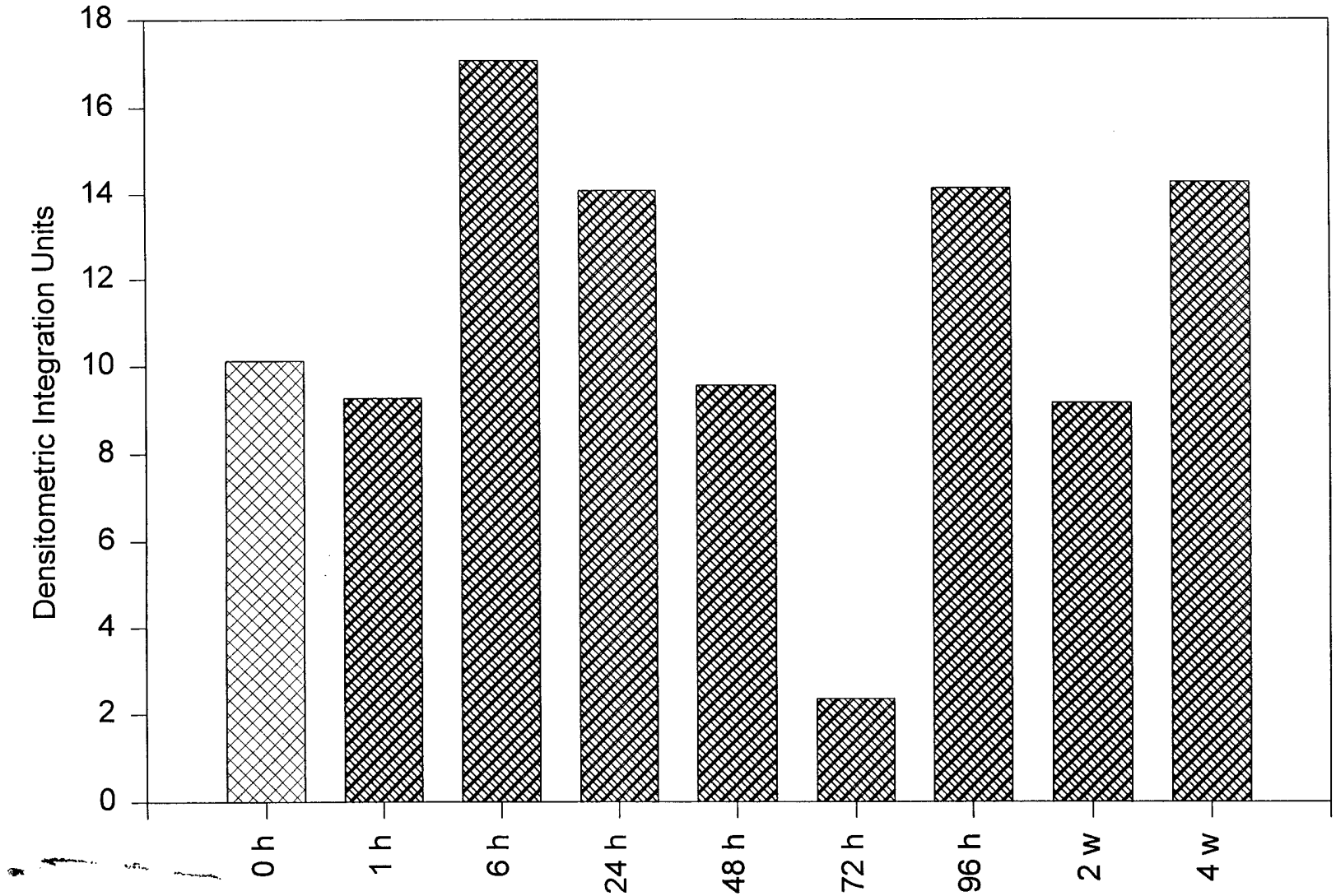
PKC-eta Levels in Patient #4 vs. Treatment with Bryostatin-1



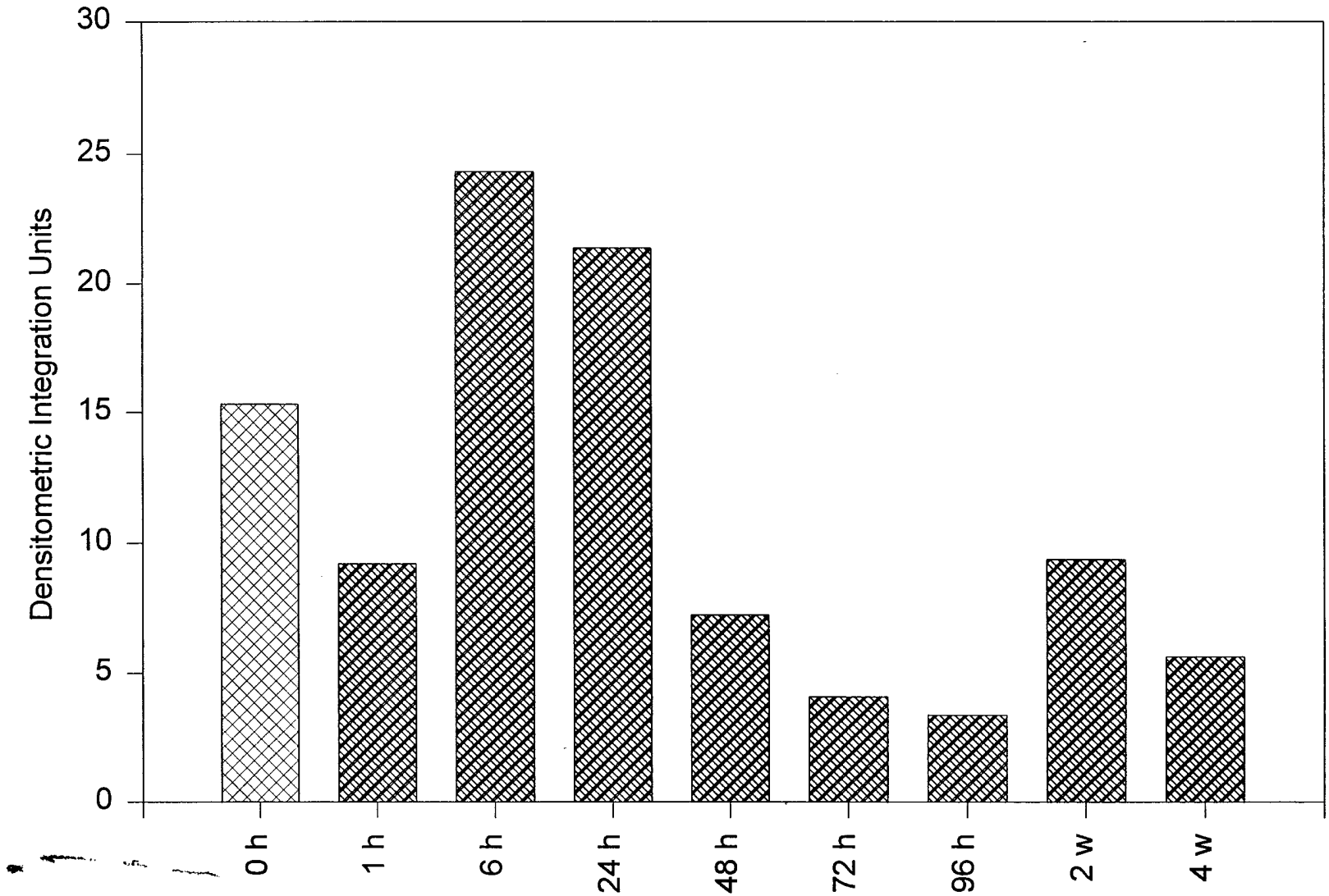
PKC-delta Levels in Patient #6 vs. Treatment with Bryostatin-1



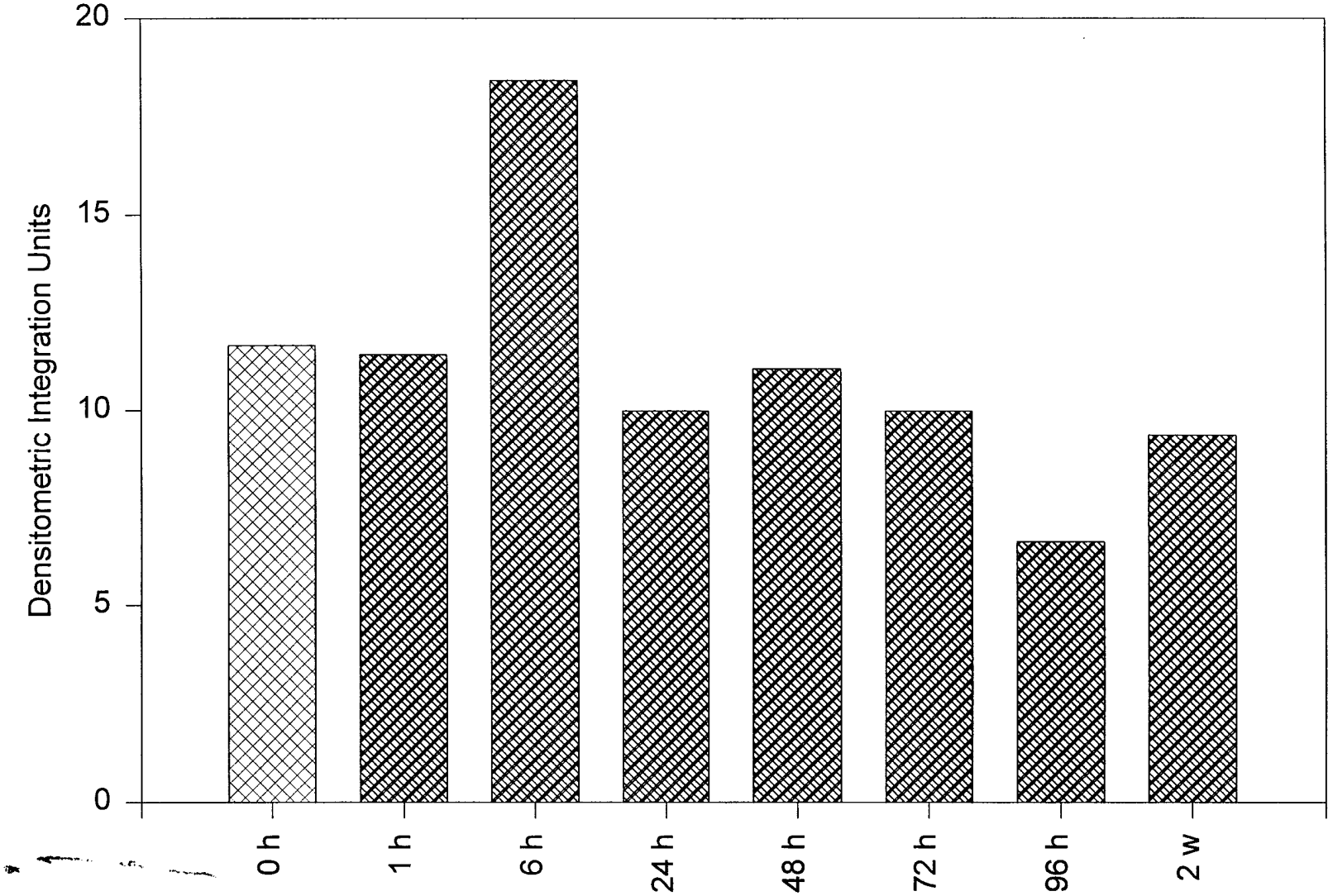
PKC-epsilon Levels in Patient #6 vs. Treatment with Bryostatin-1



PKC-eta Levels vs. Treatment with Bryostatin-1



PKC-delta Levels in Patient #7 vs. Treatment with Bryostatin-1



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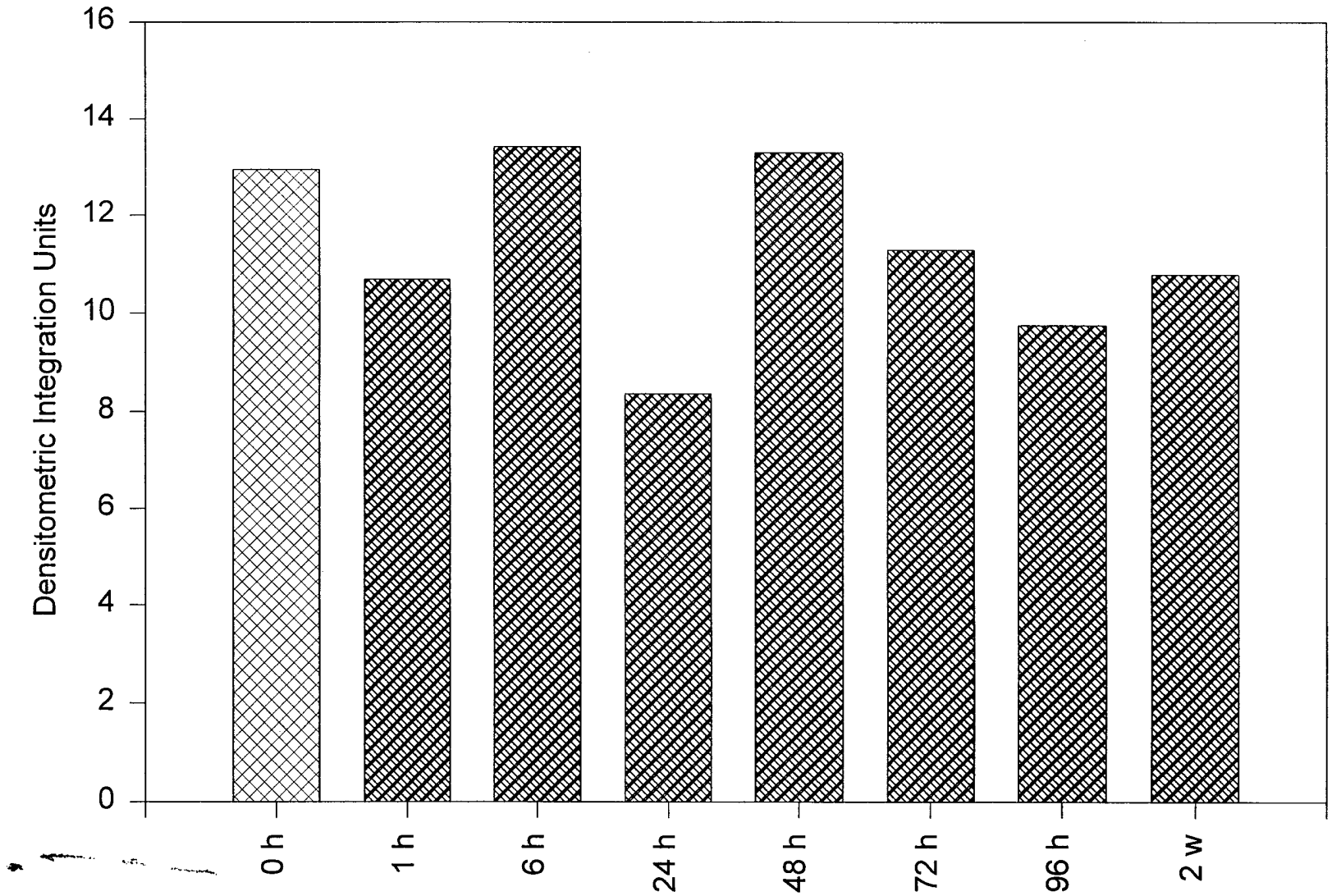
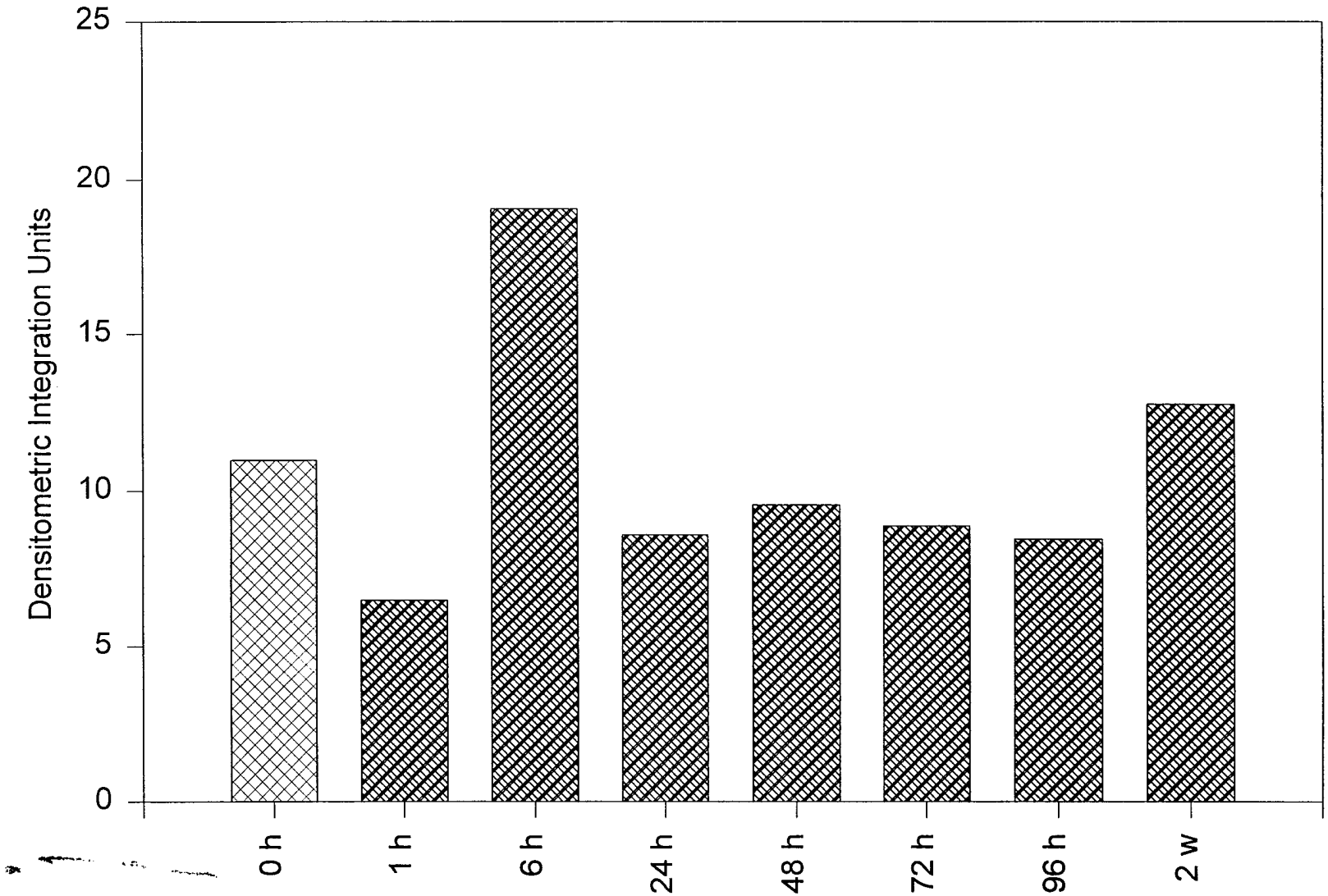
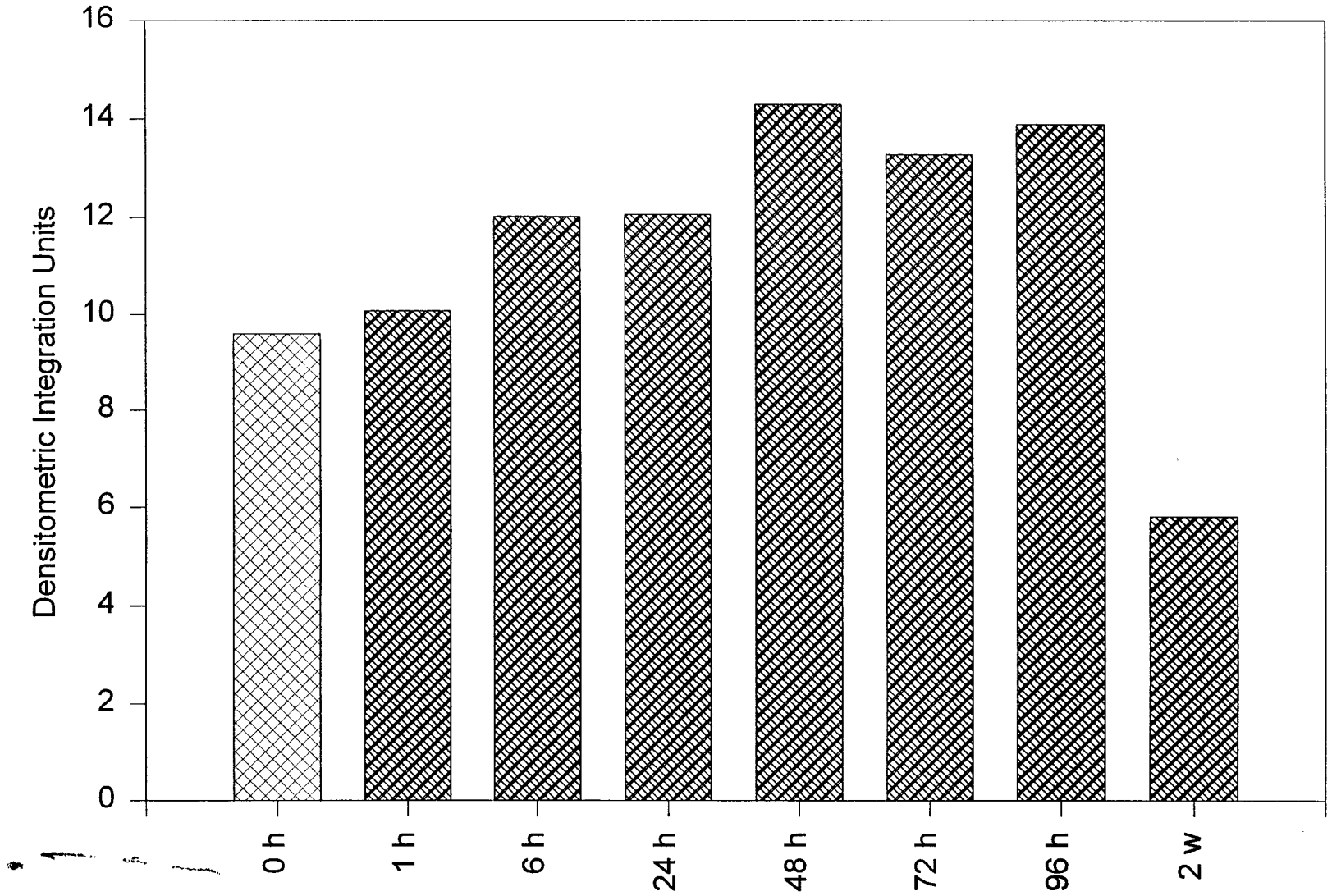


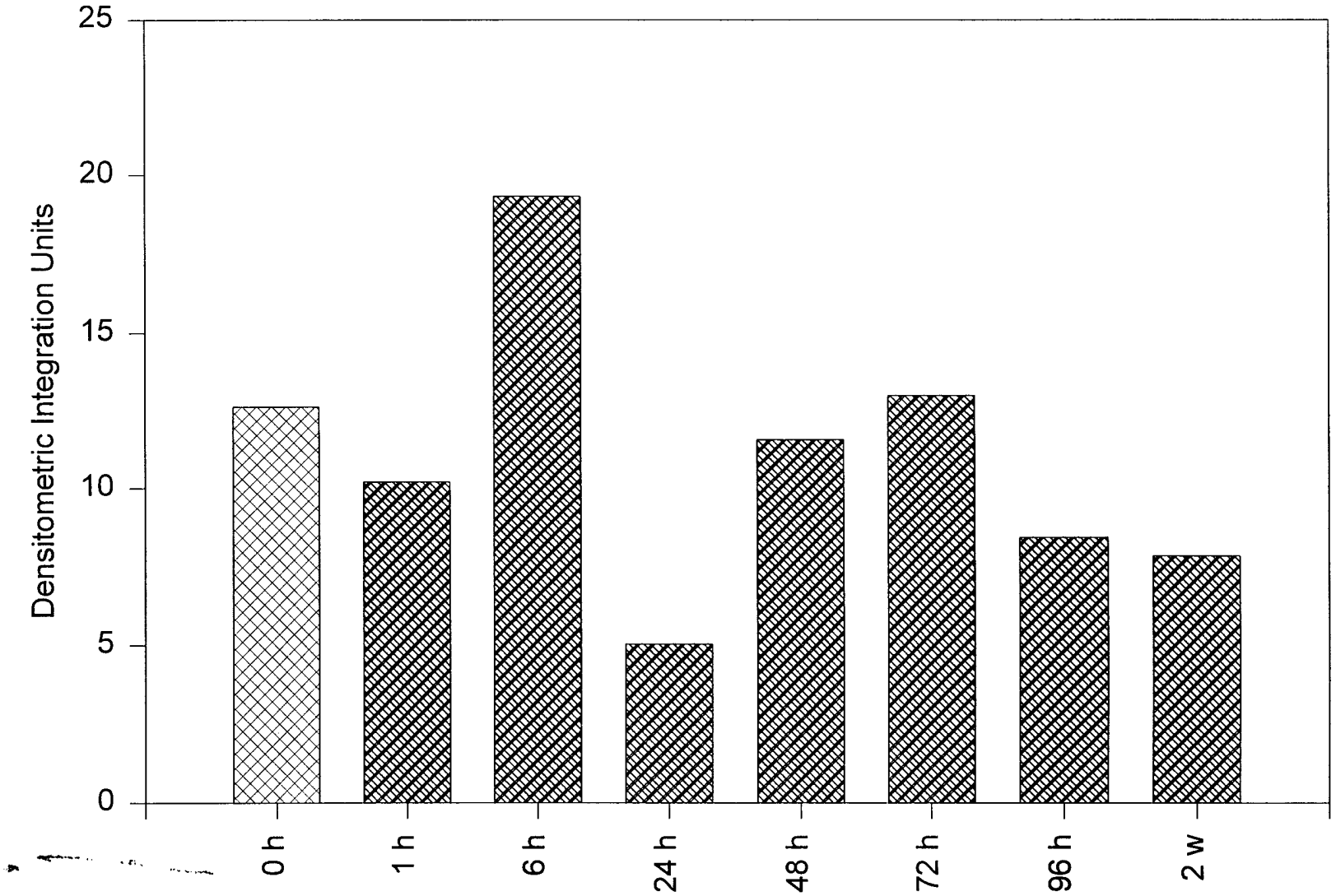
PKC-eta Levels in Patient #7 vs. Treatment with Bryostatin-1



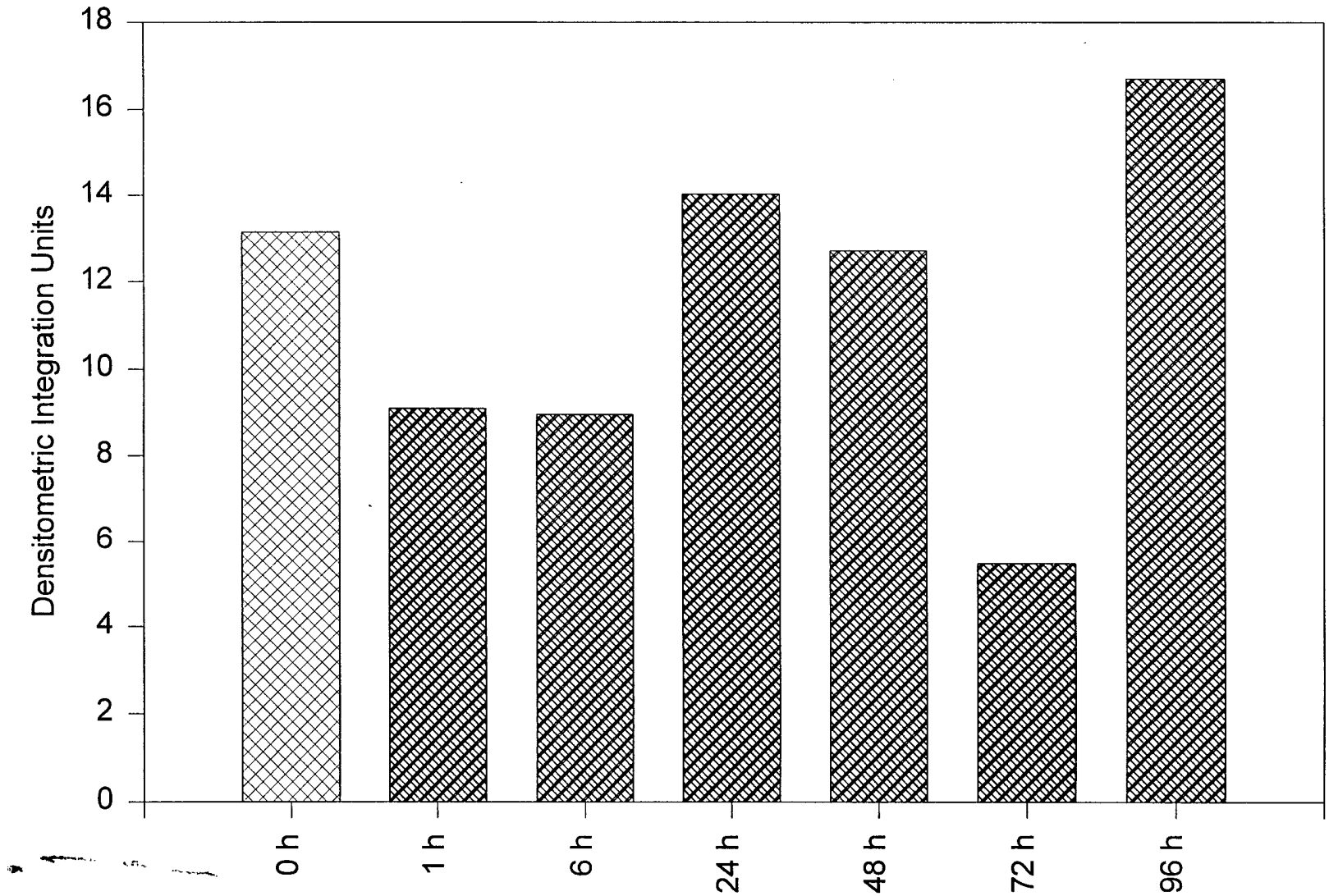
PKC-gamma Levels in Patient #7 vs. Treatment with Bryostatin-1



PKC-mu Levels in Patient #7 vs. Treatment with Bryostatin-1



PKC-alpha Levels in Patient #8 vs. Treatment with Bryostatin-1



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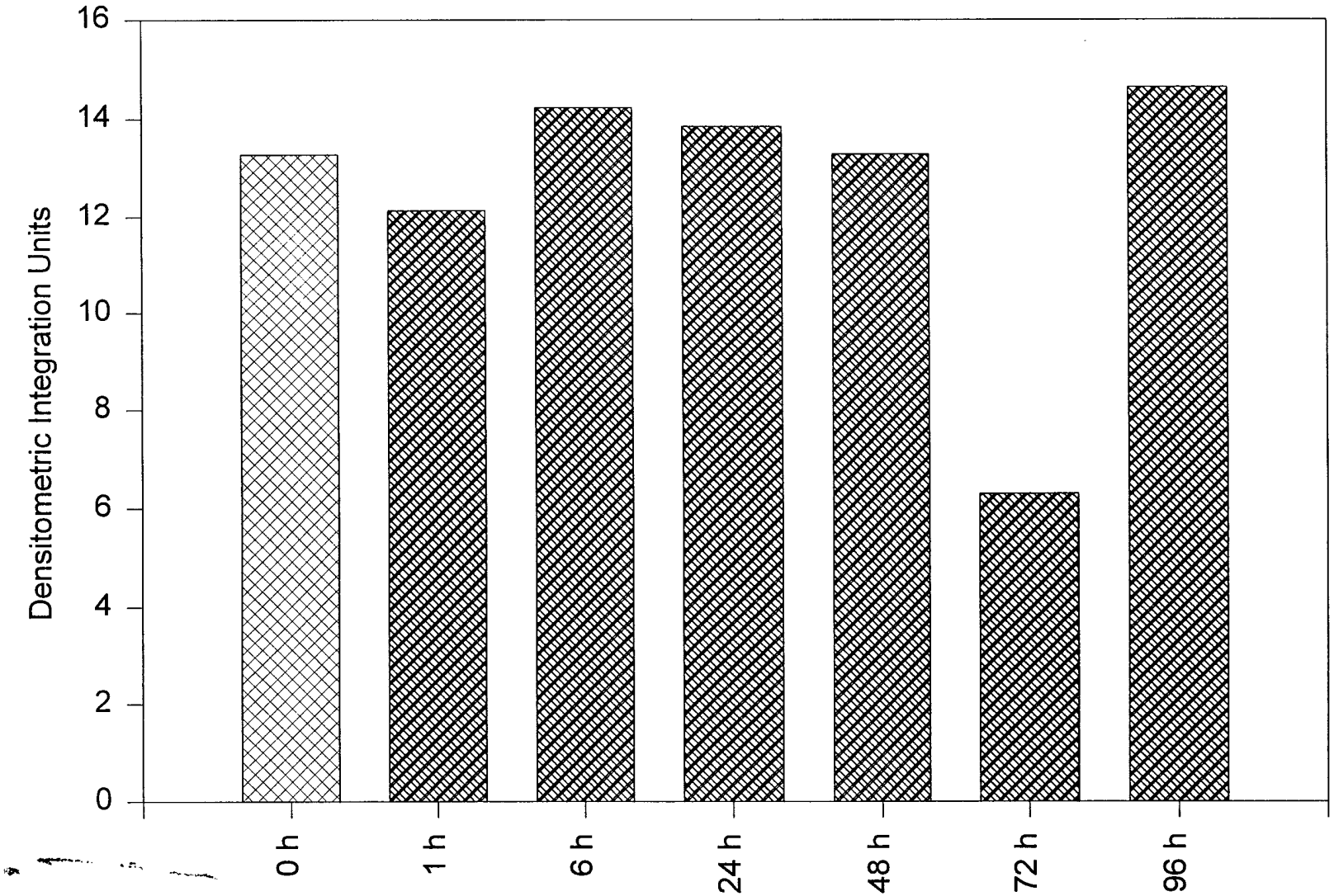

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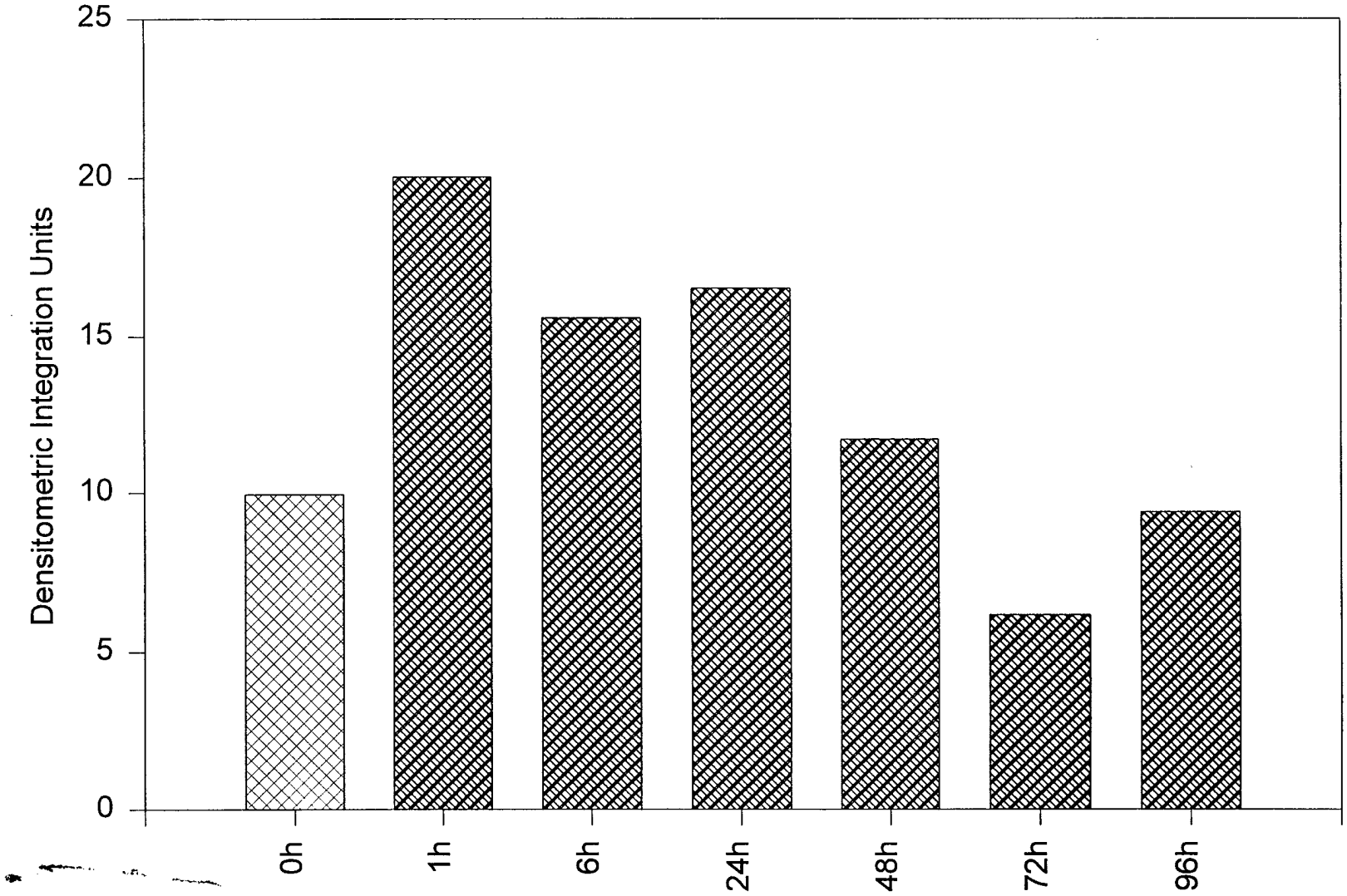

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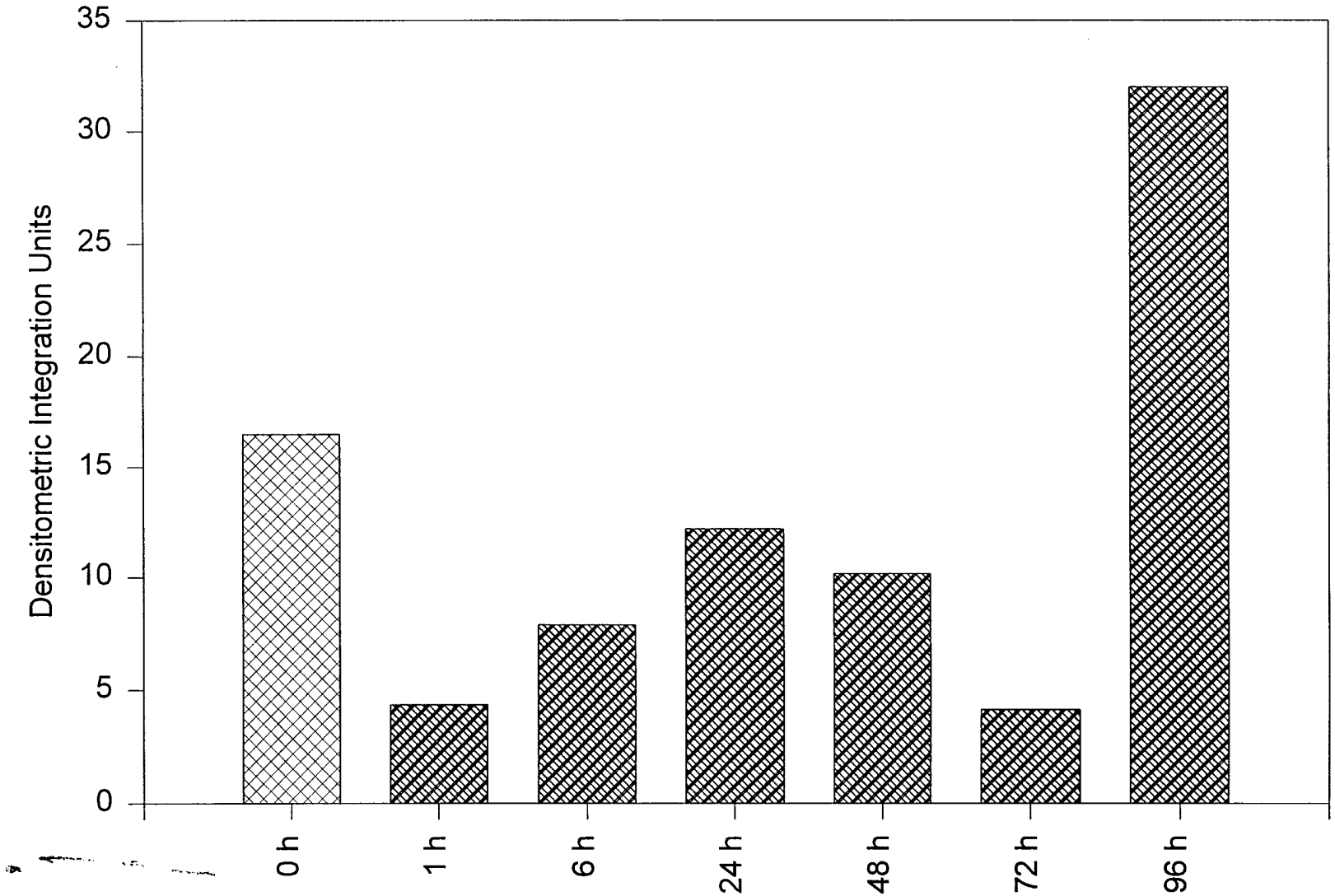

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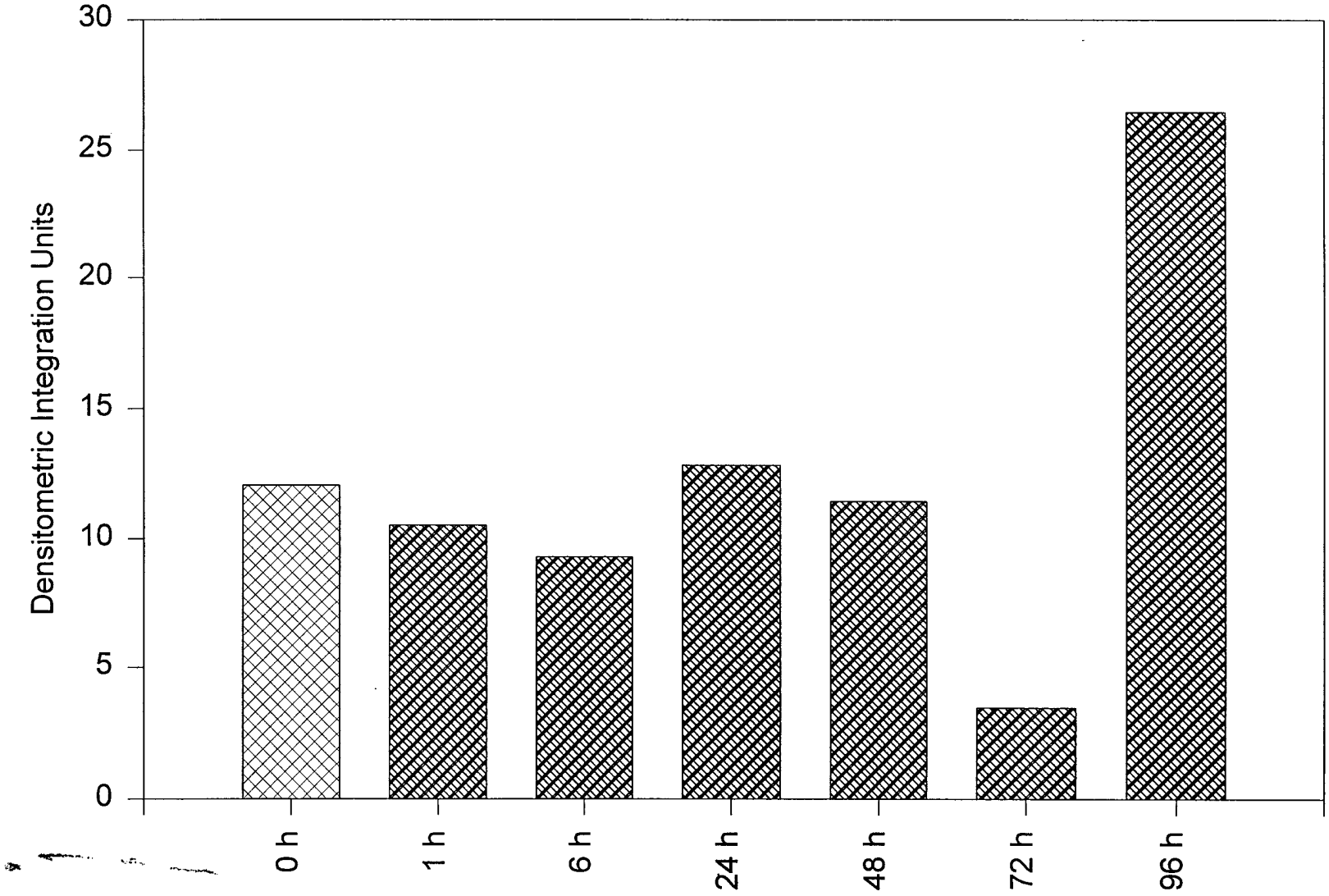

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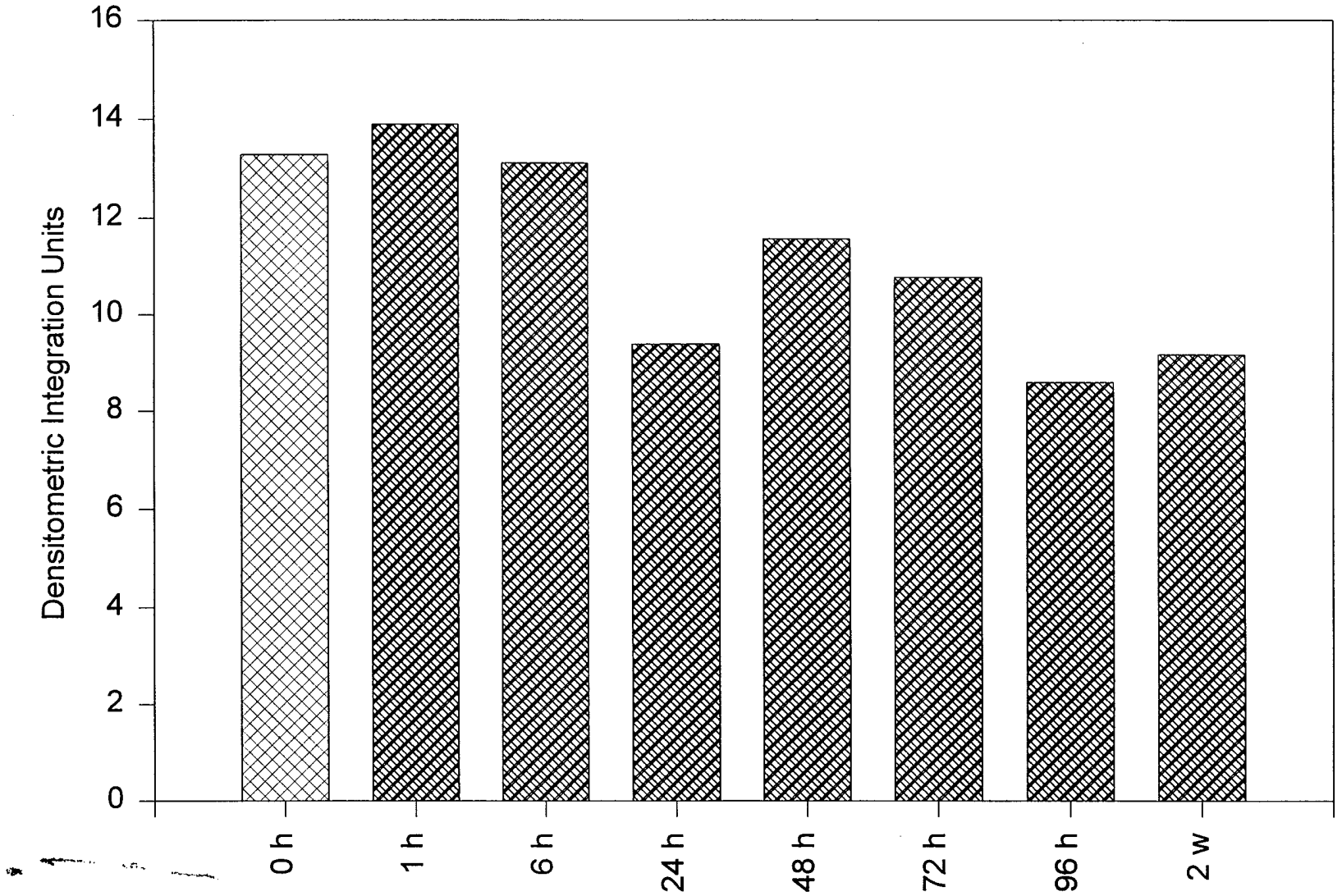

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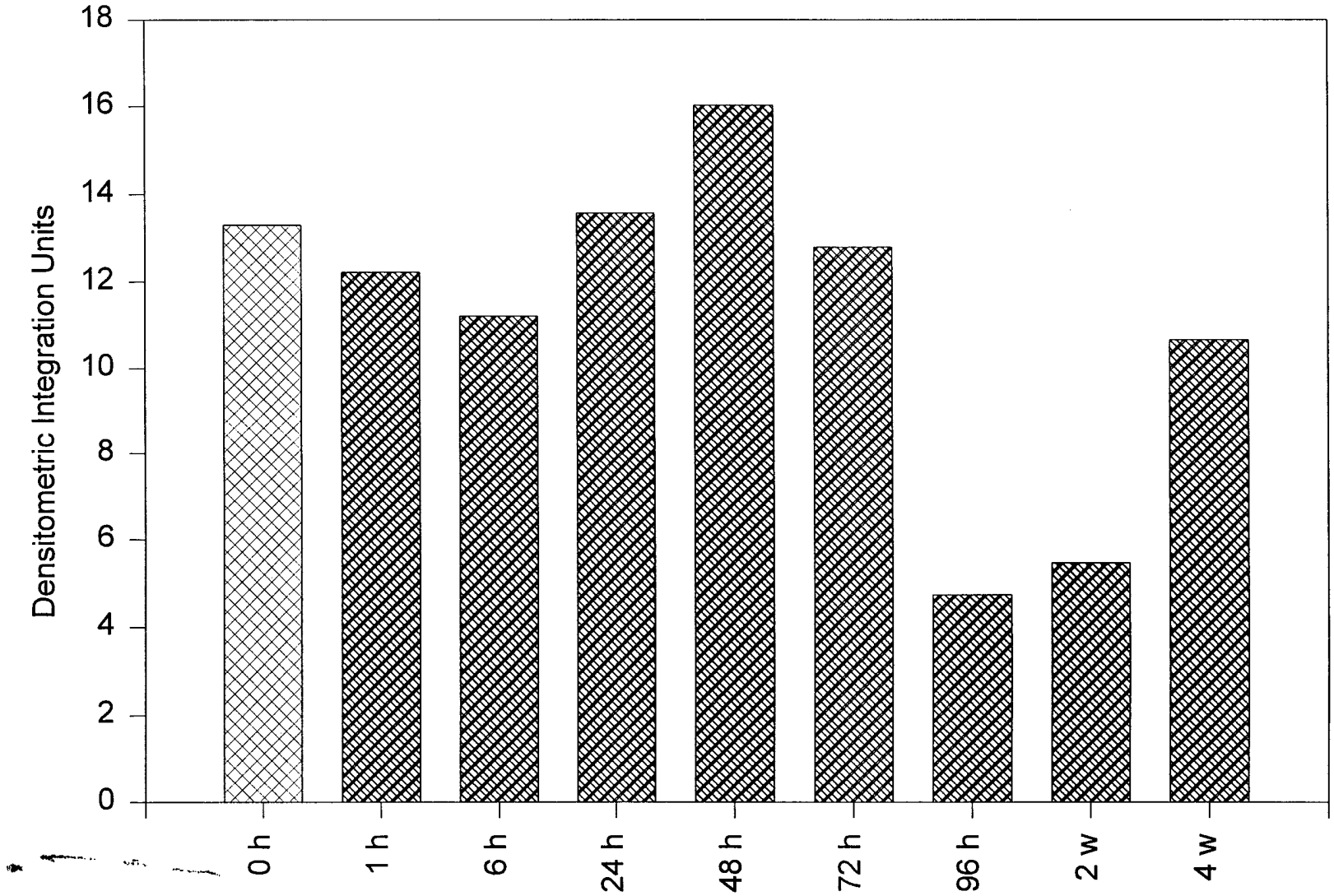

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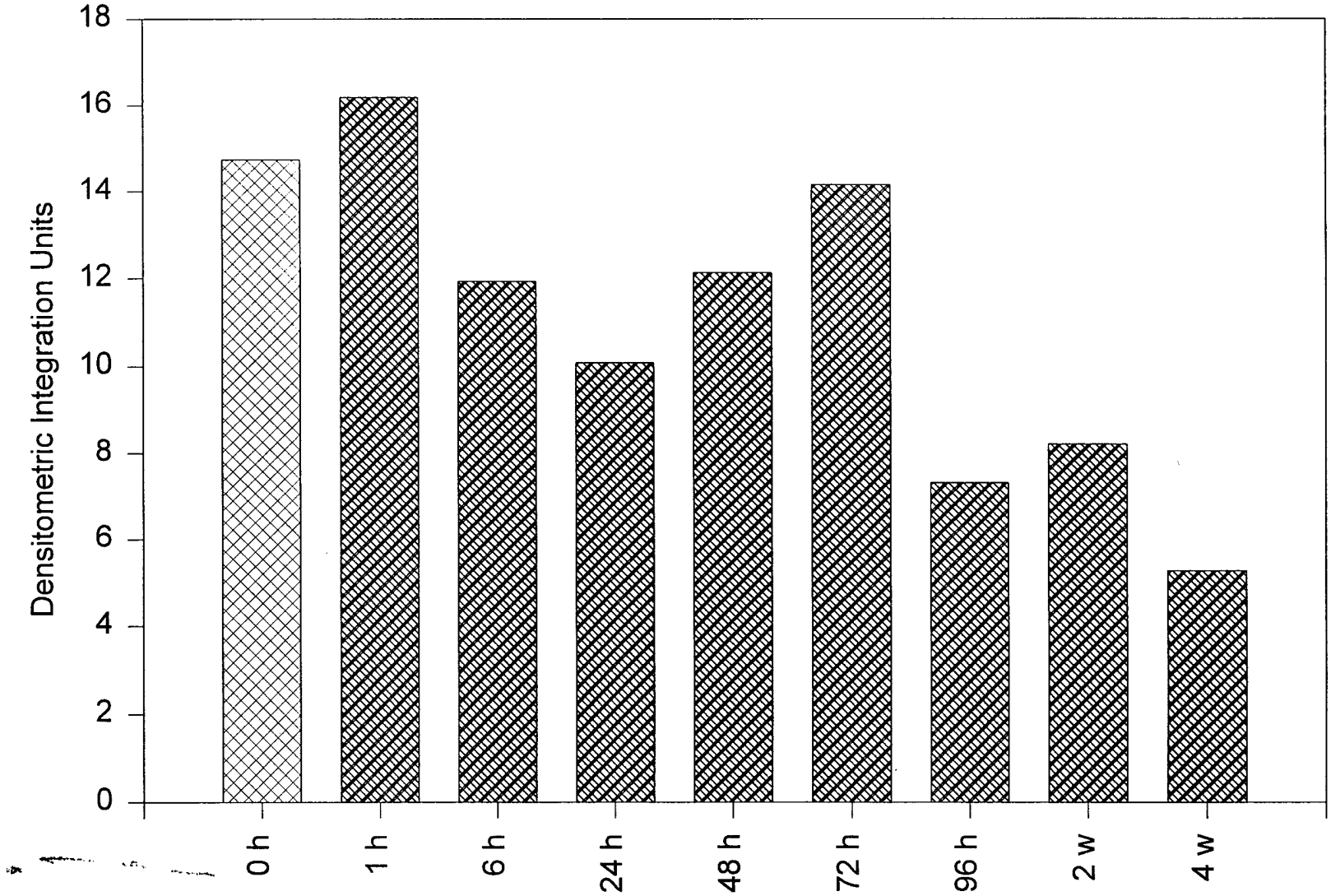

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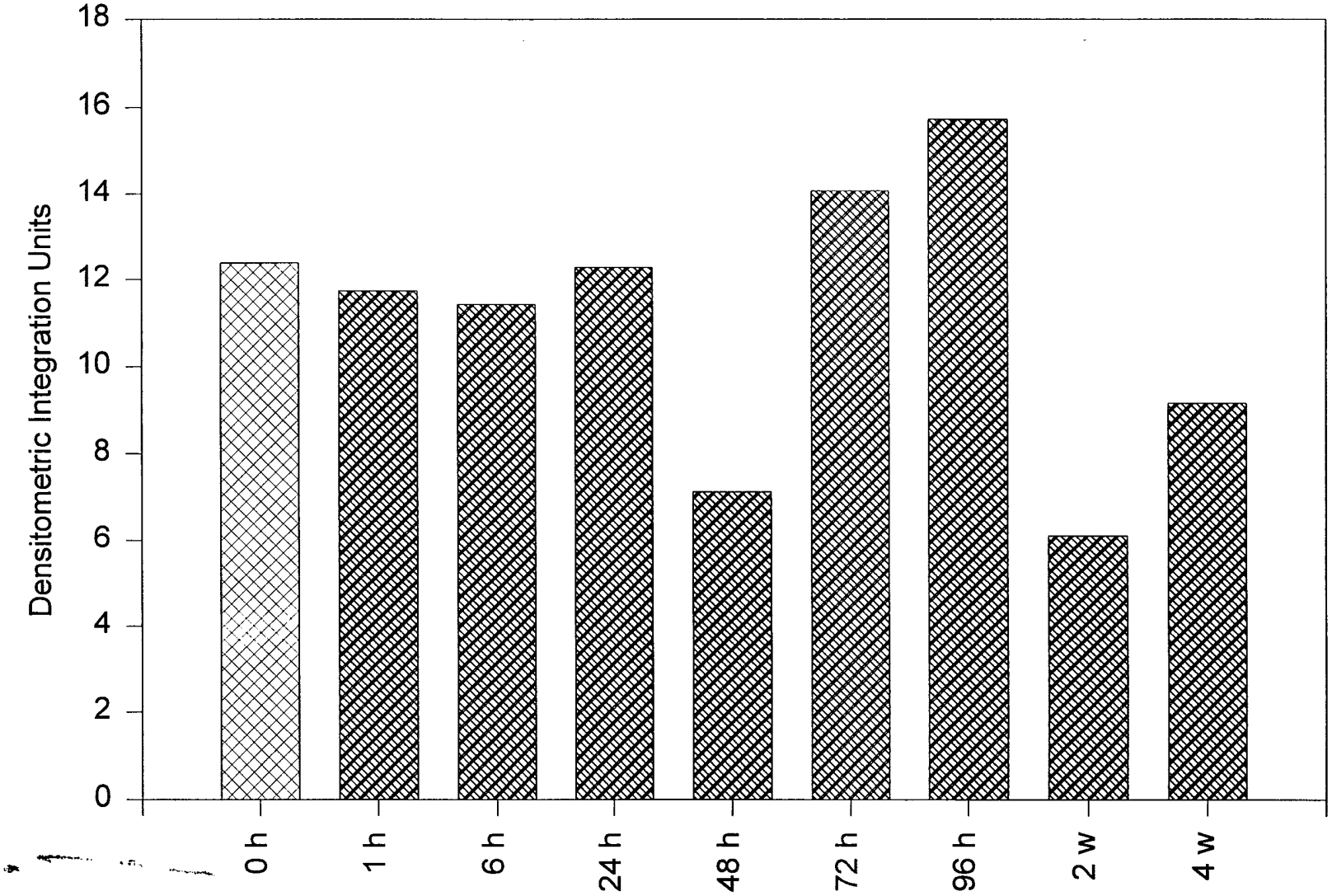

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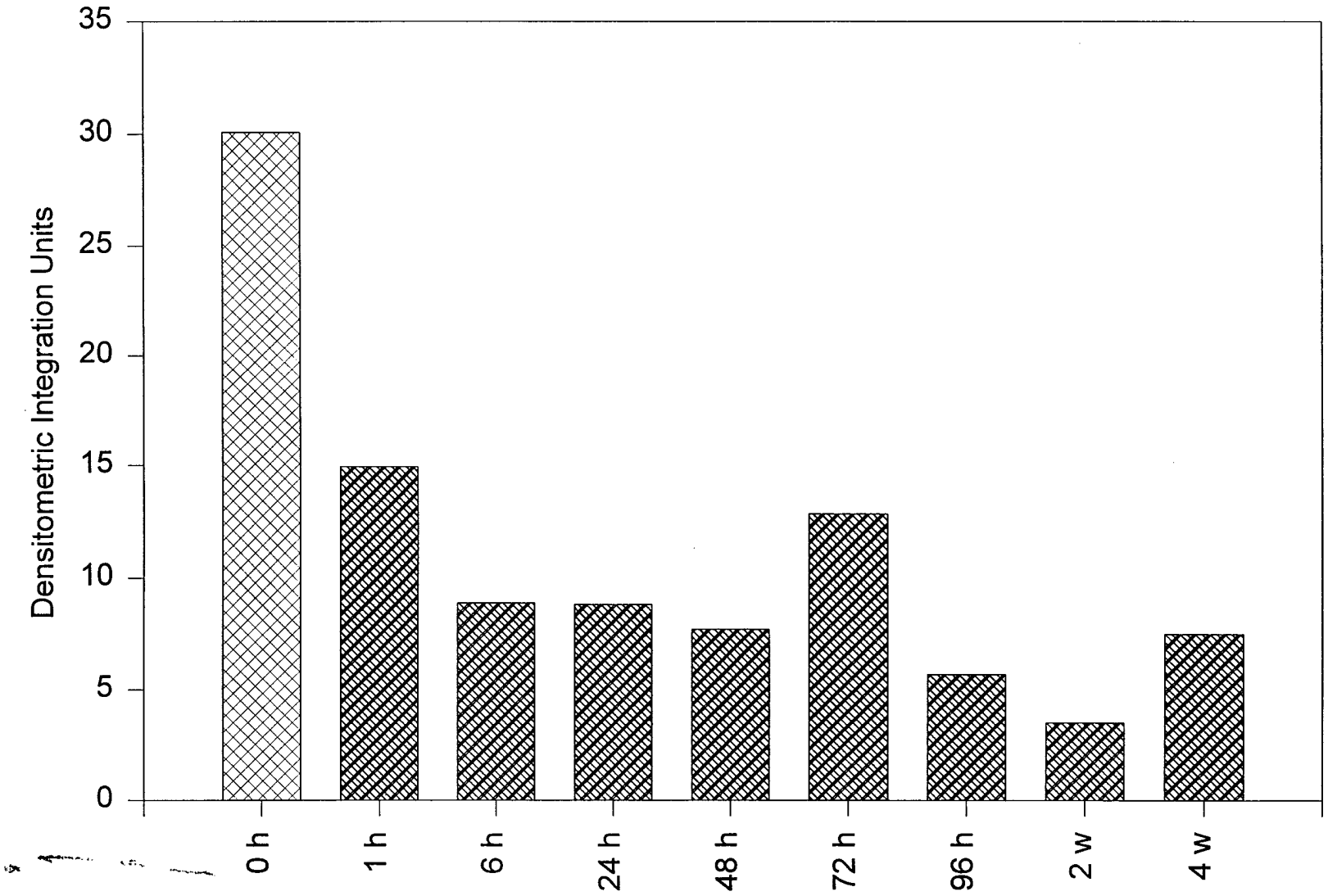

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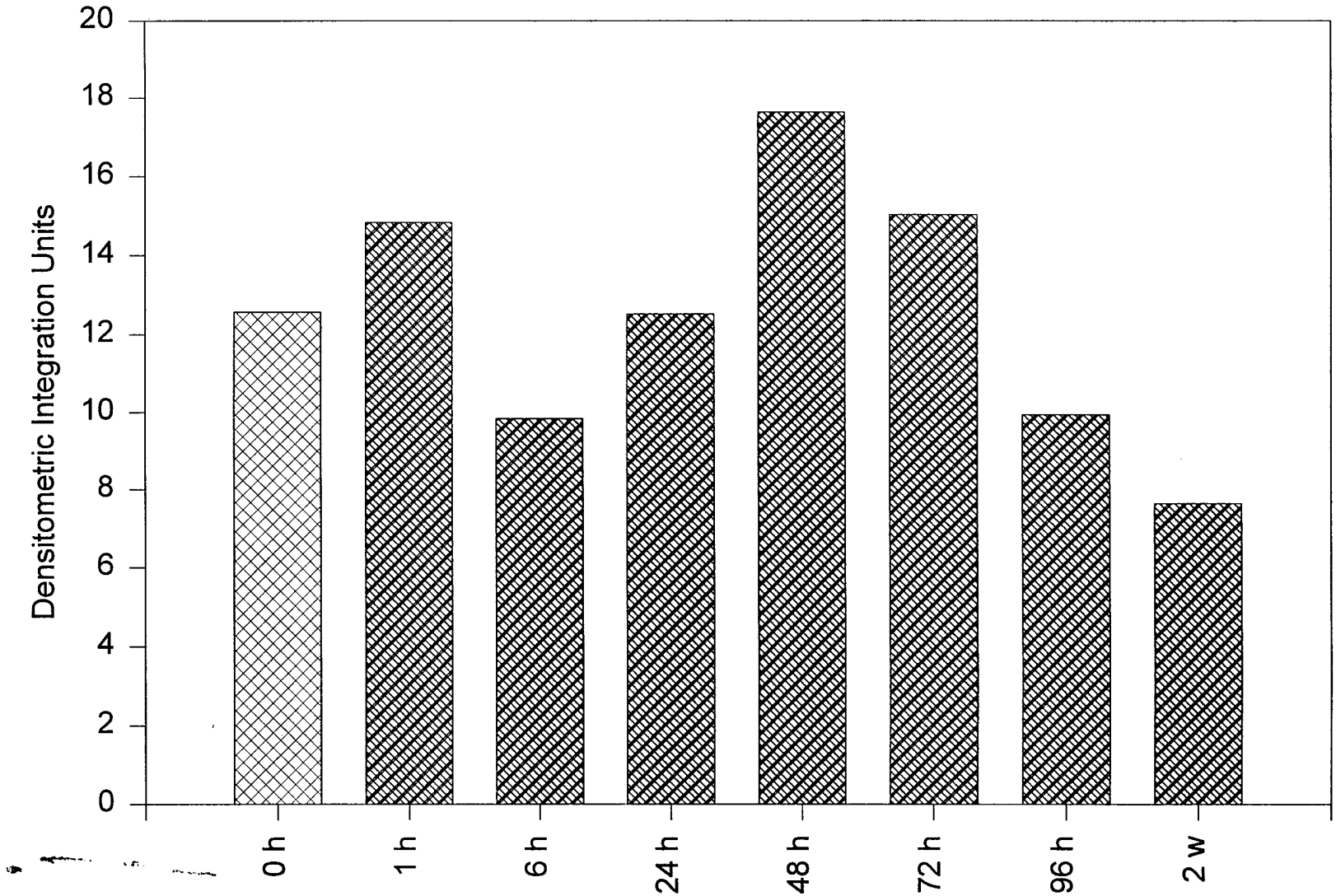

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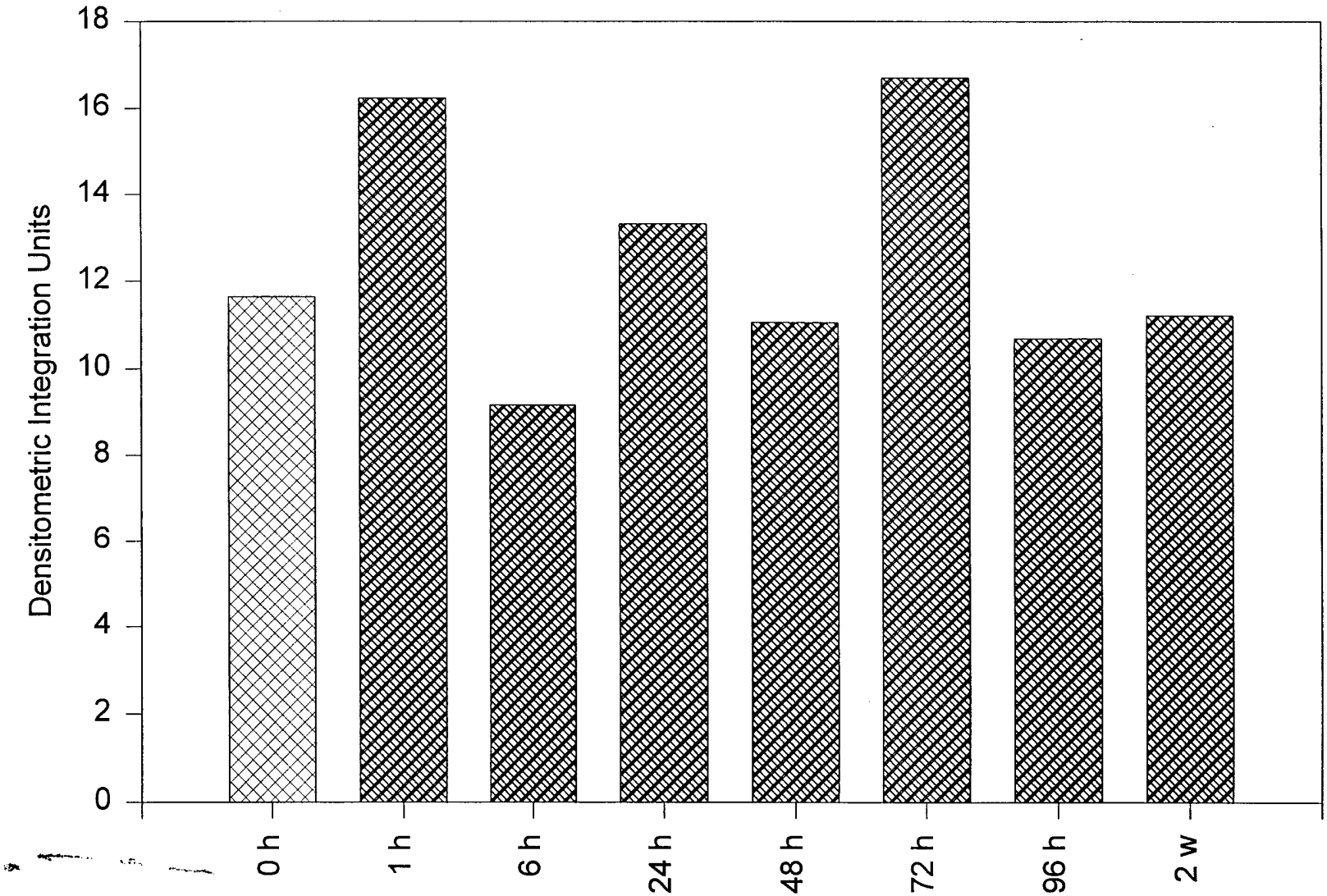

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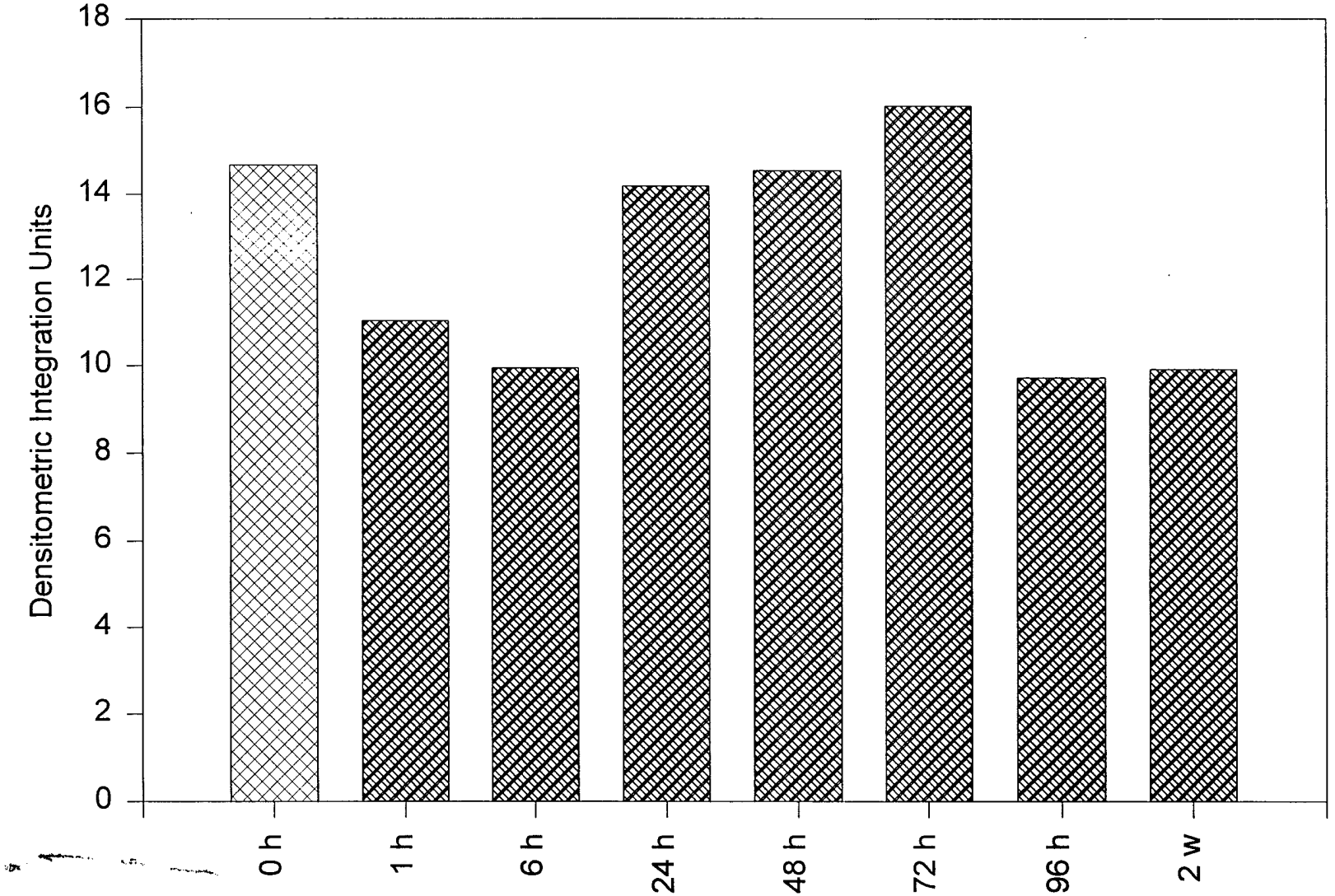

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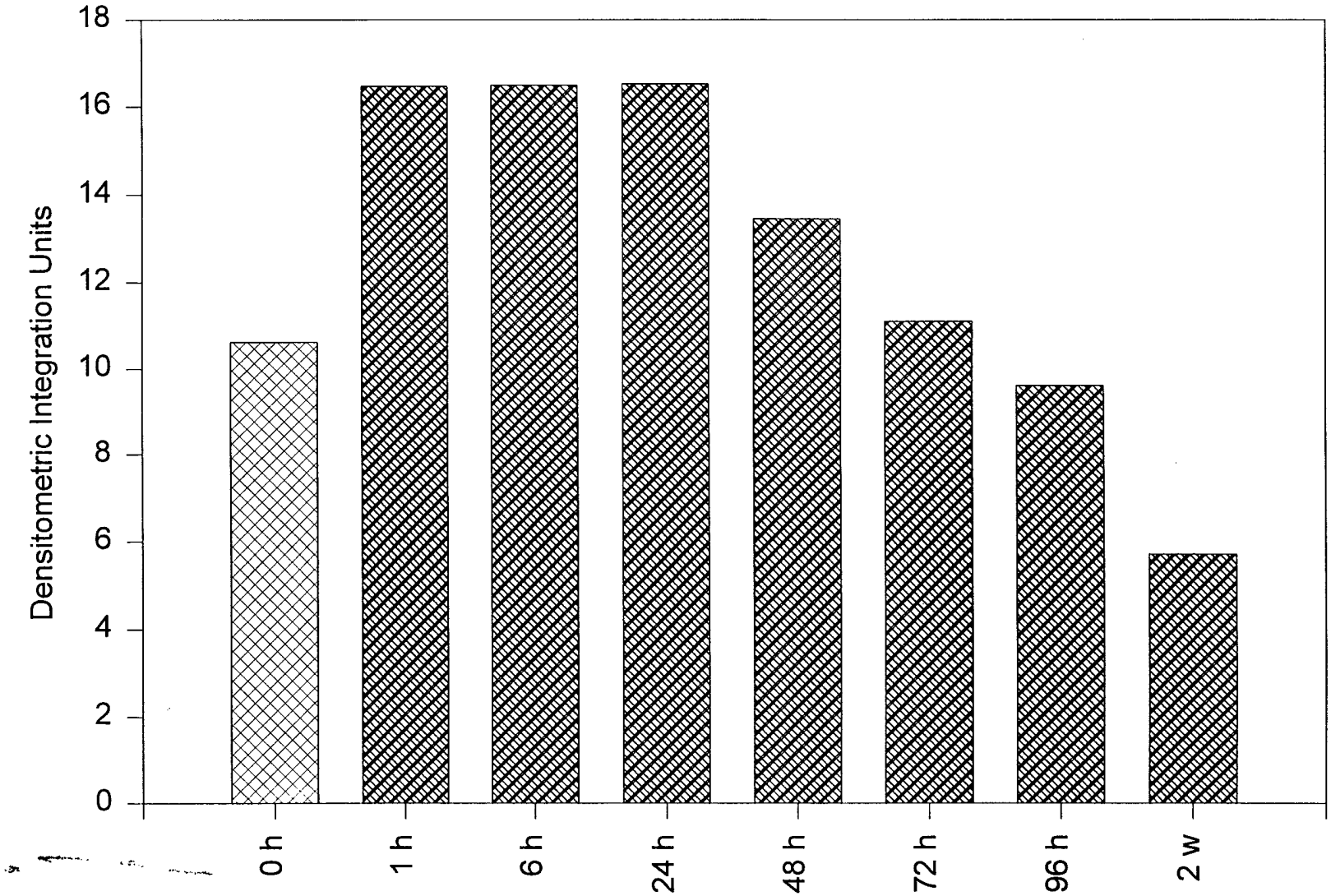

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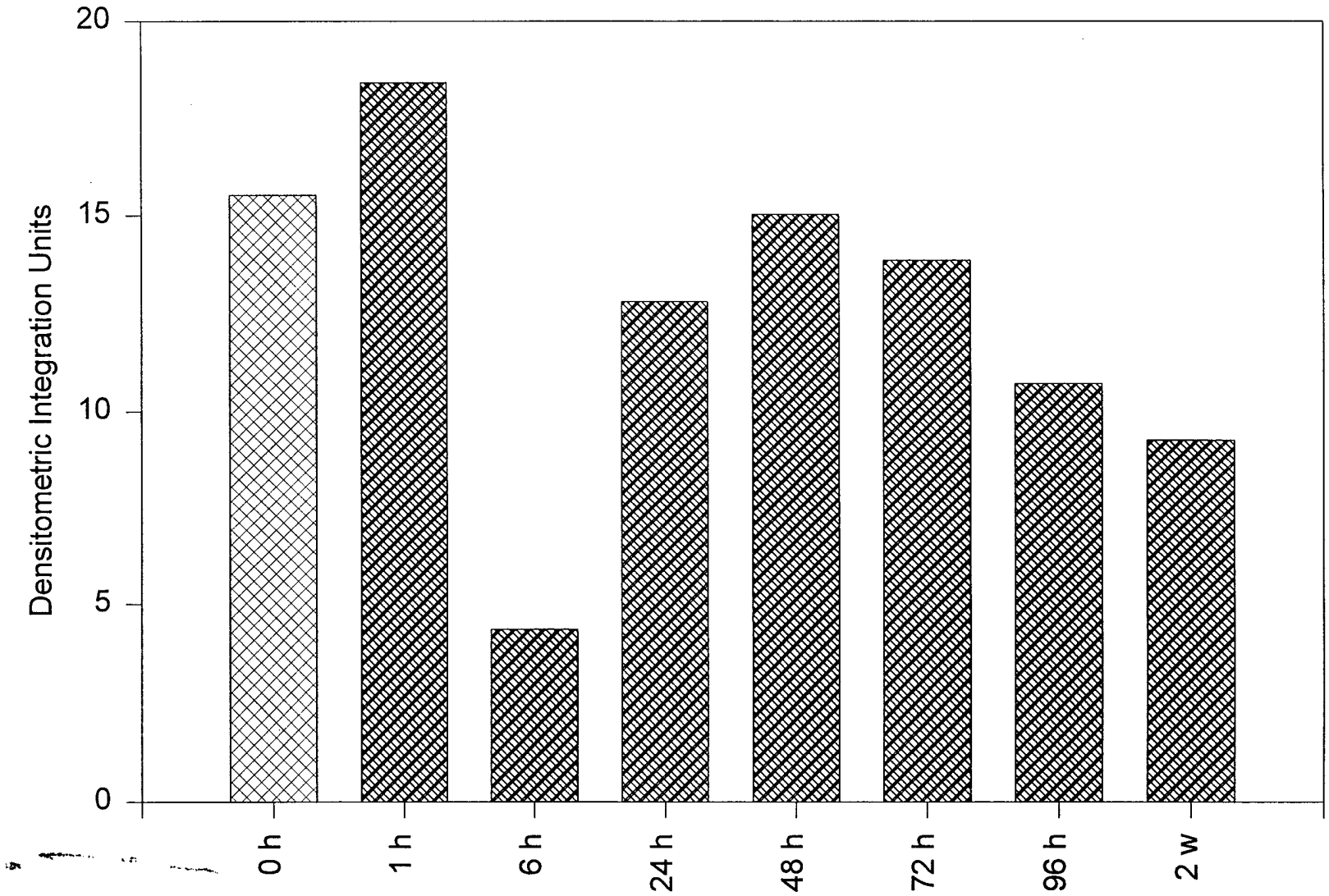

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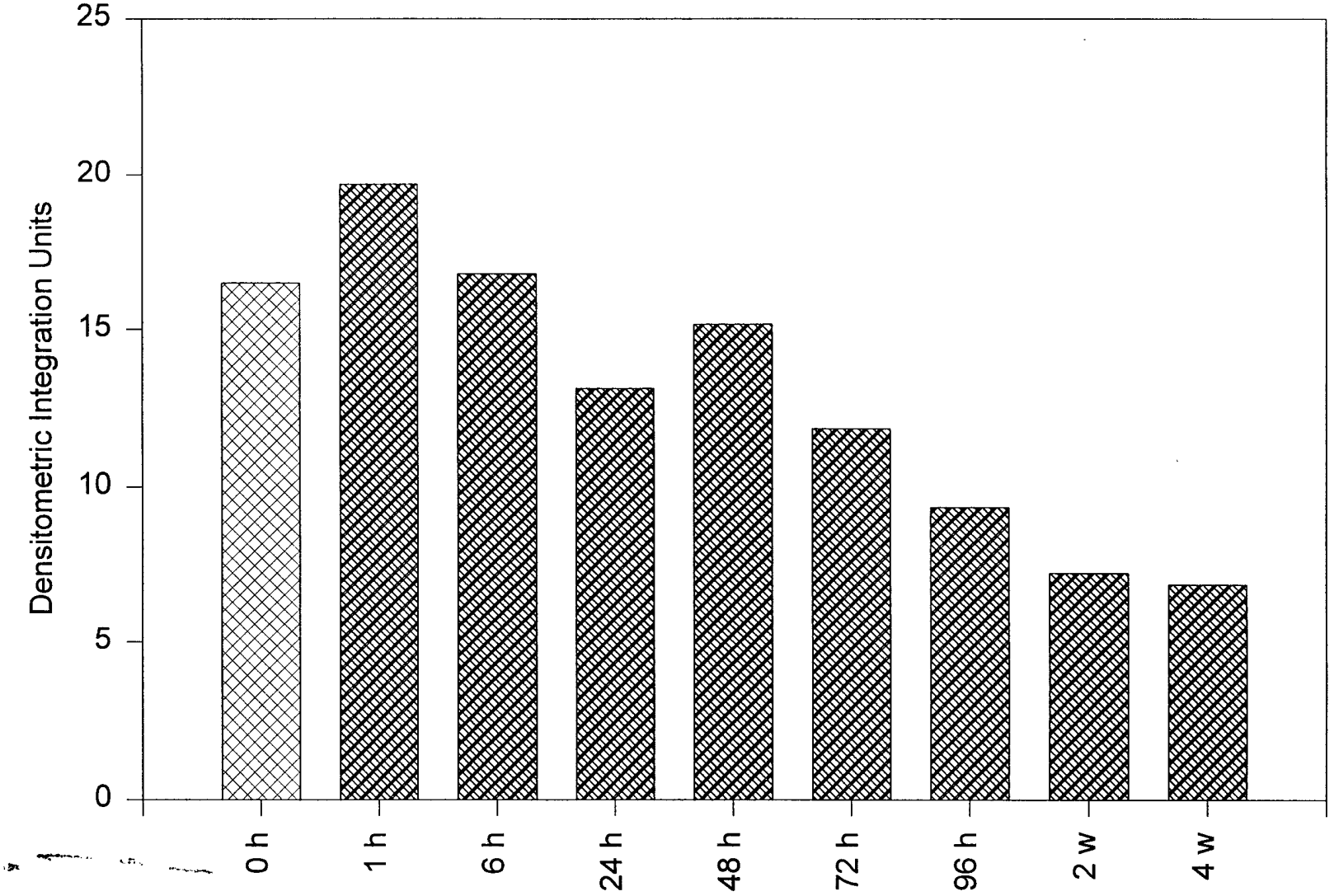
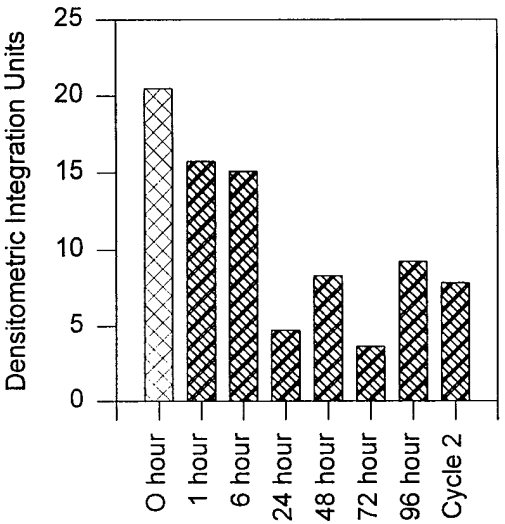


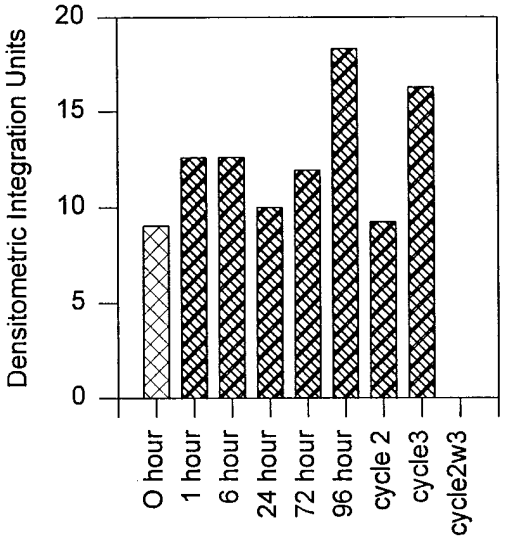
Patient #11: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha



Patient #12: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha



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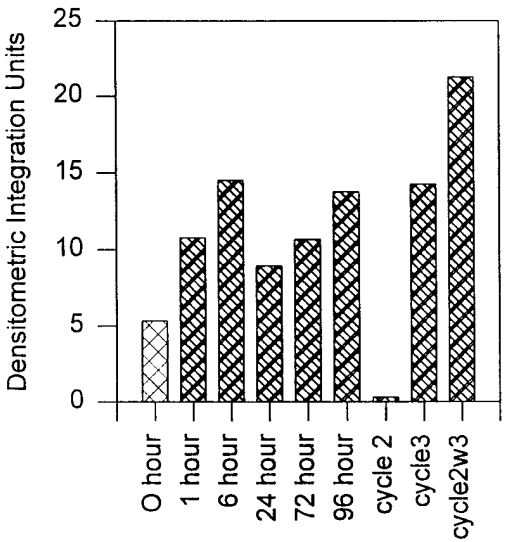

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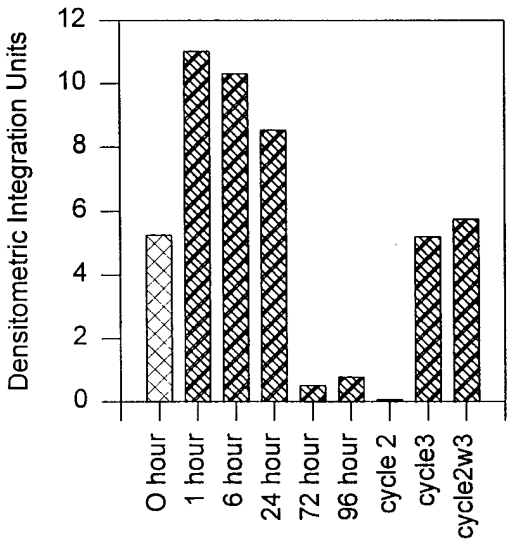
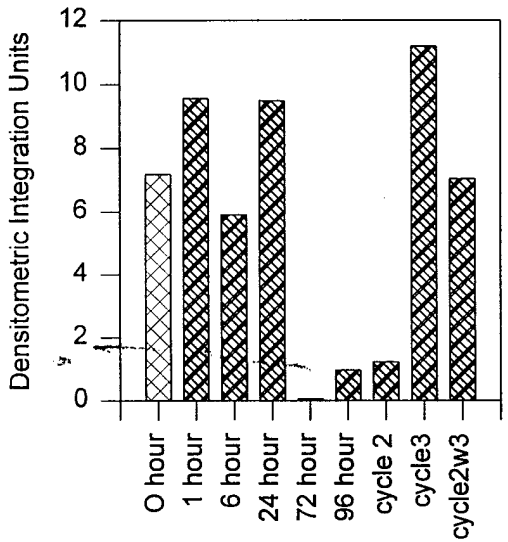
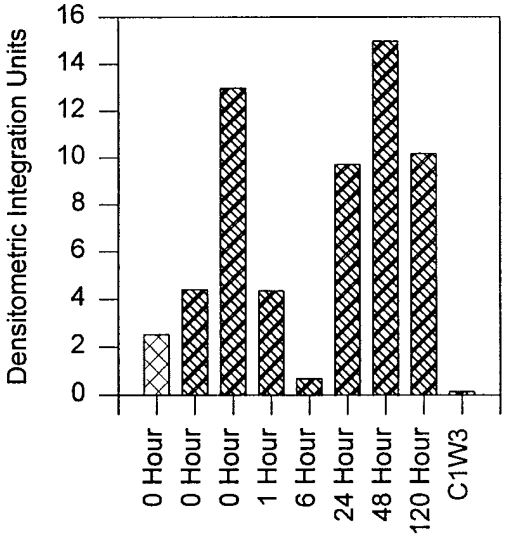


PKC-gamma

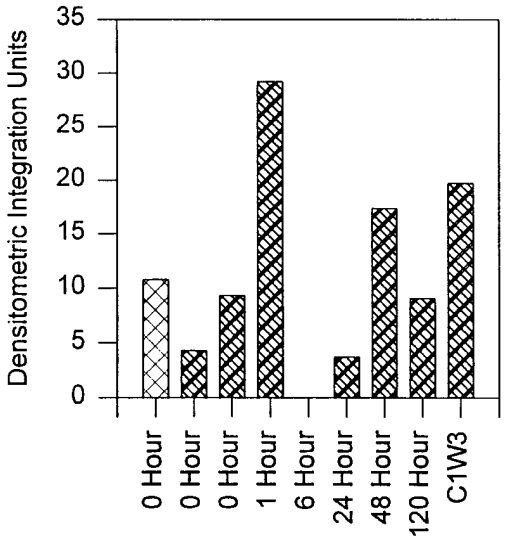


Patient #15: Protein Kinase C Levels vs. Treatment with Bryostatin-1

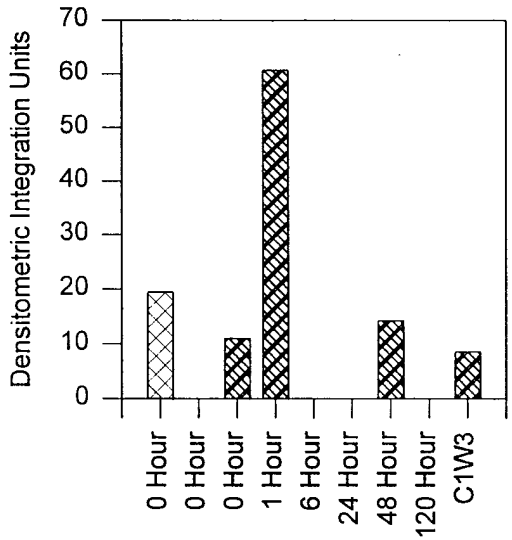
PKC-eta



PKC-alpha

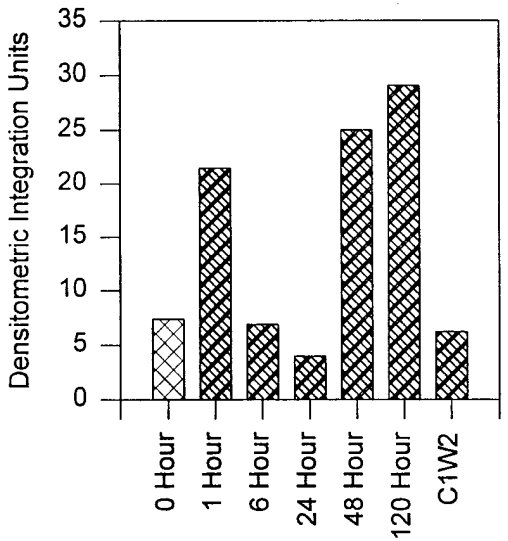


PKC-gamma

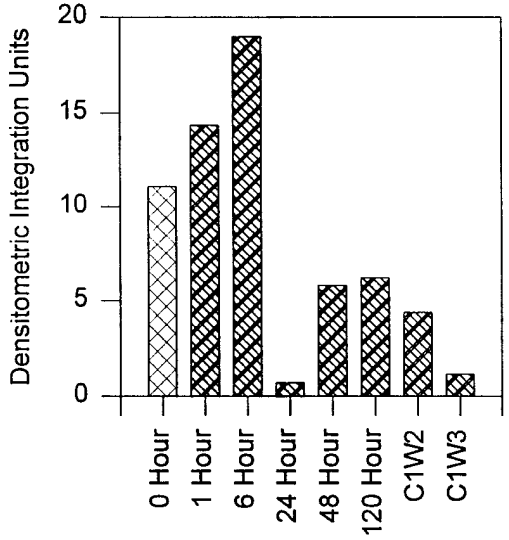


Patient #16: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha

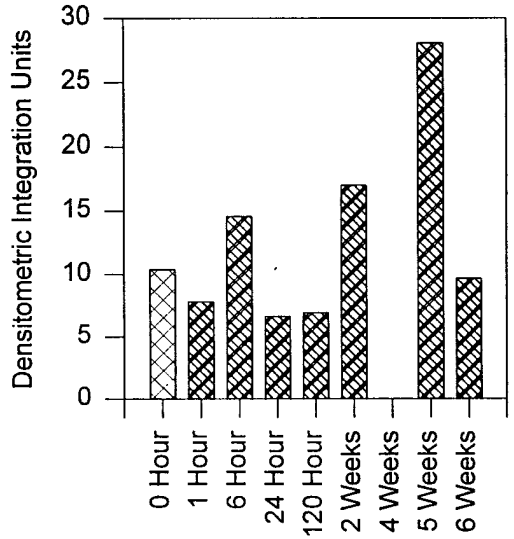


PKC-eta

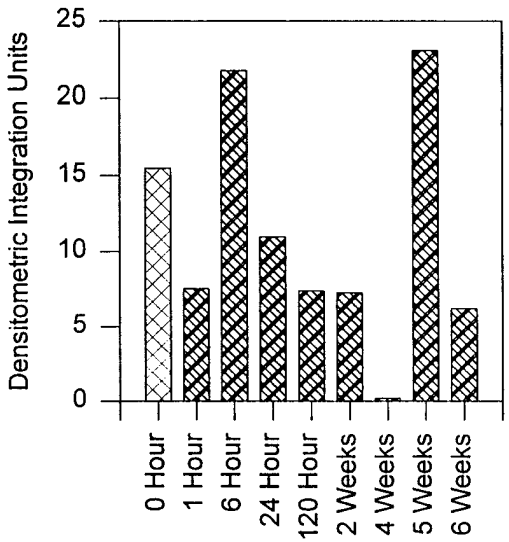


Patient #17: Protein Kinase C Levels vs. Treatment with Bryostatin-1

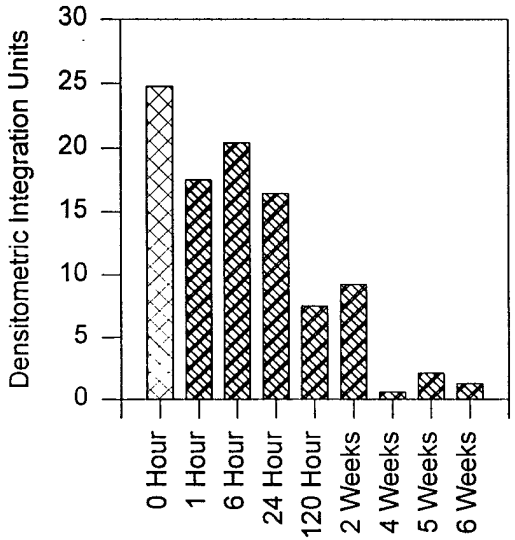
PKC-alpha



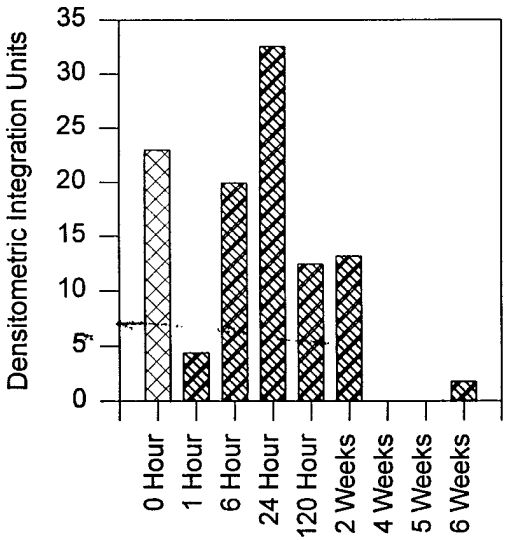
PKC-delta



PKC-epsilon

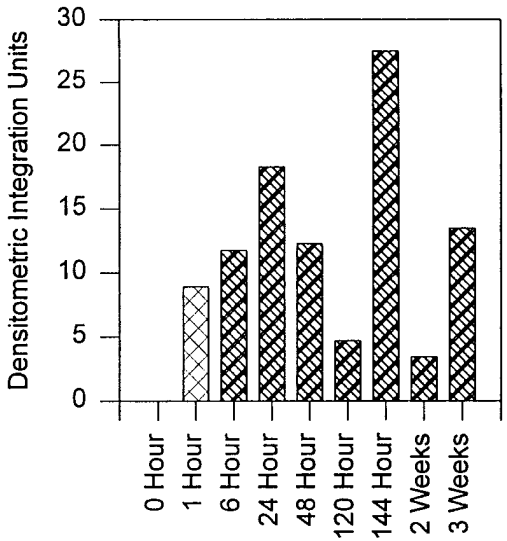


PKC-eta



Patient #18: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha



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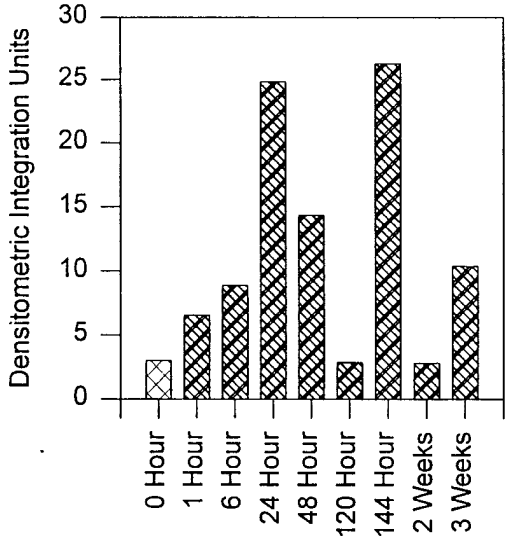
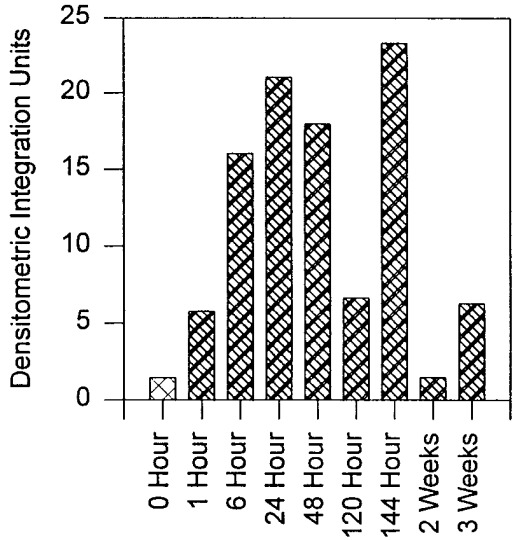
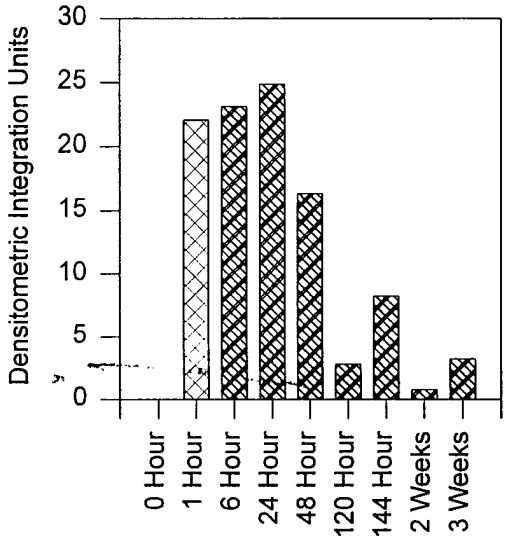


PKC-epsilon

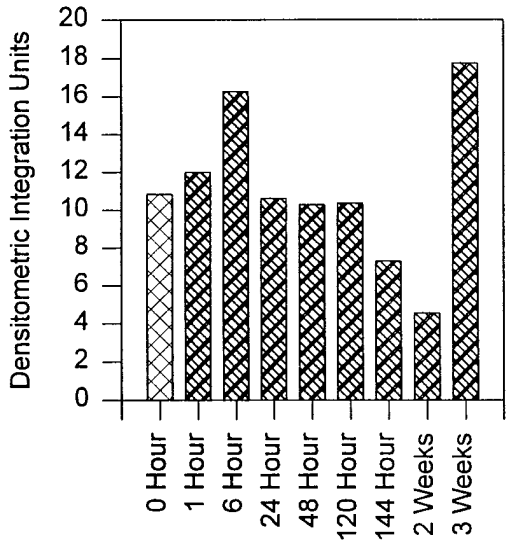


PKC-eta

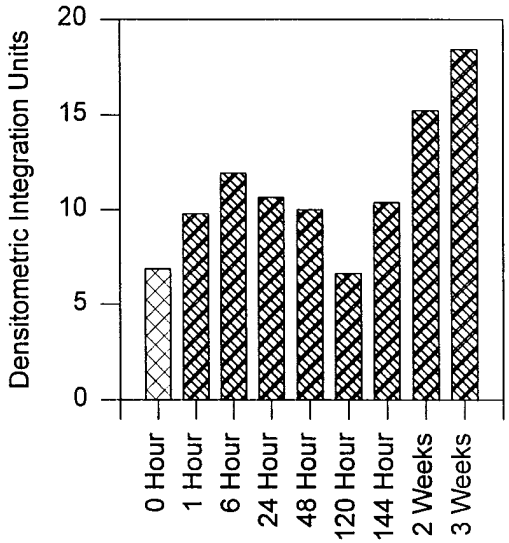


Patient 19: Protein Kinase C Levels vs. Treatment with Bryostatin-1

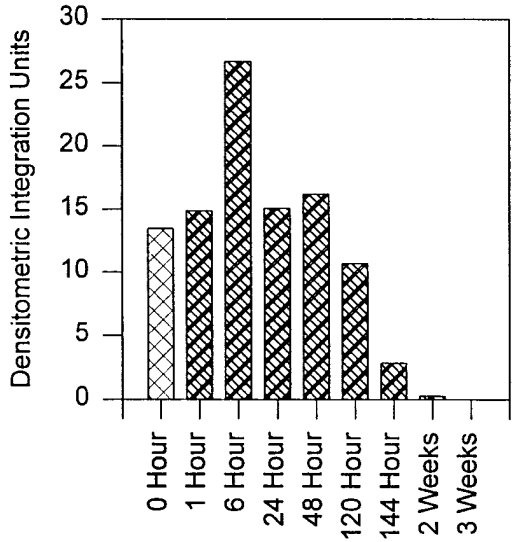
PKC-alpha



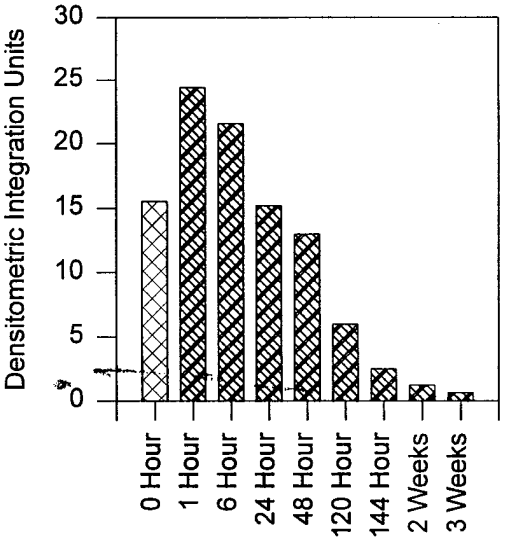
PKC-delta



PKC-epsilon

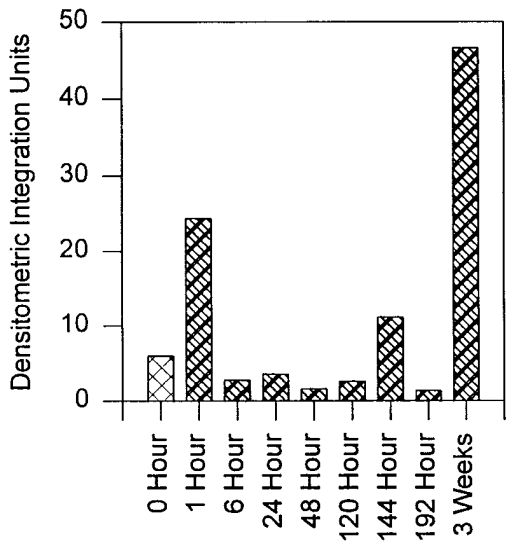


PKC-eta

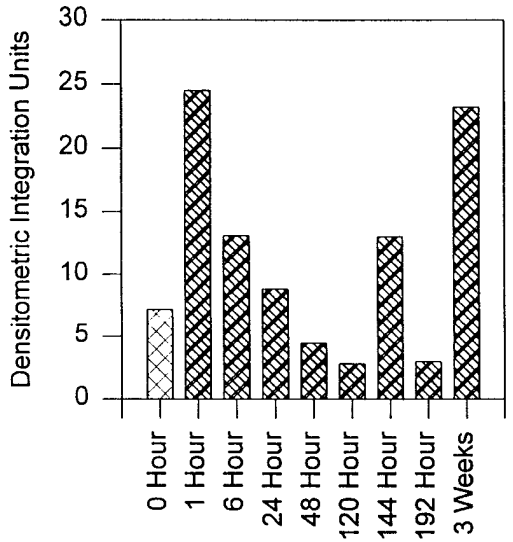


Patient #22: Protein Kinase C Levels vs. Treatment with Bryostatin-1

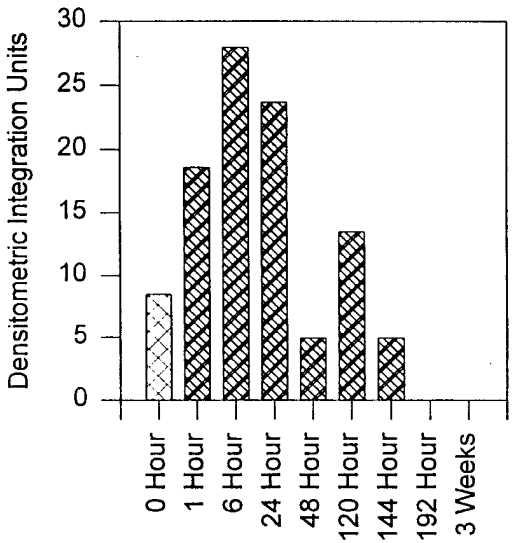
PKC-alpha



PKC-delta

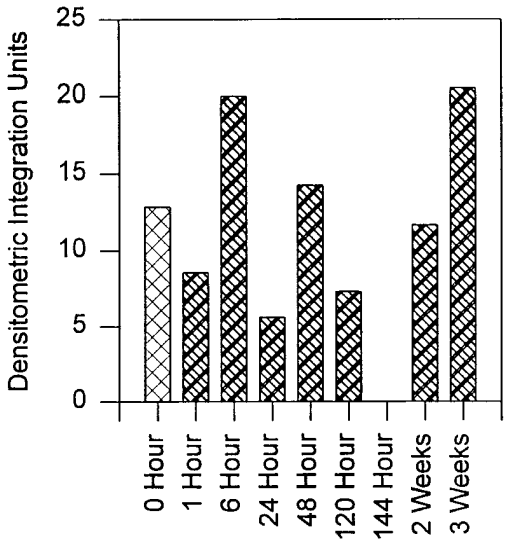


PKC-eta

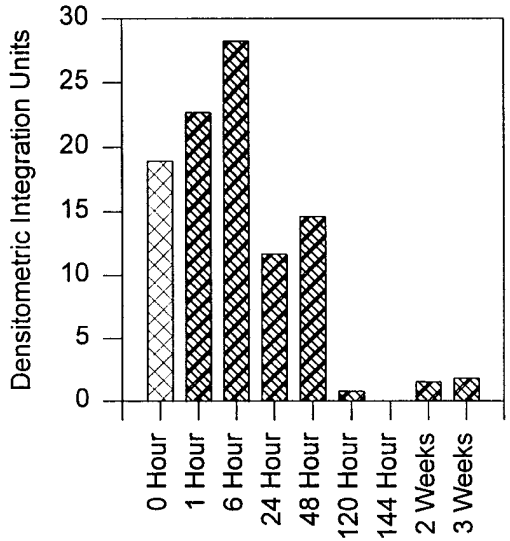


Patient #23: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha

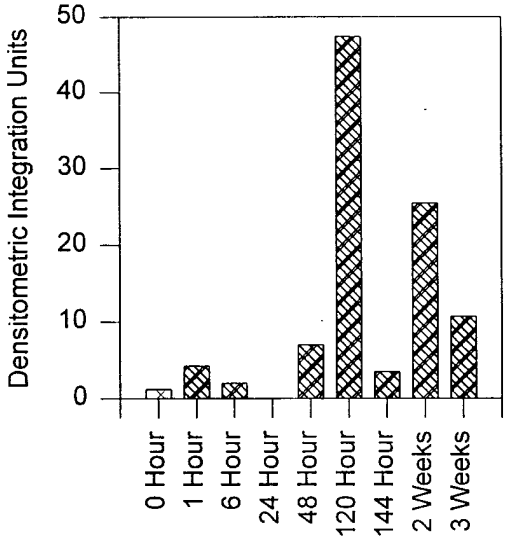


PKC-eta

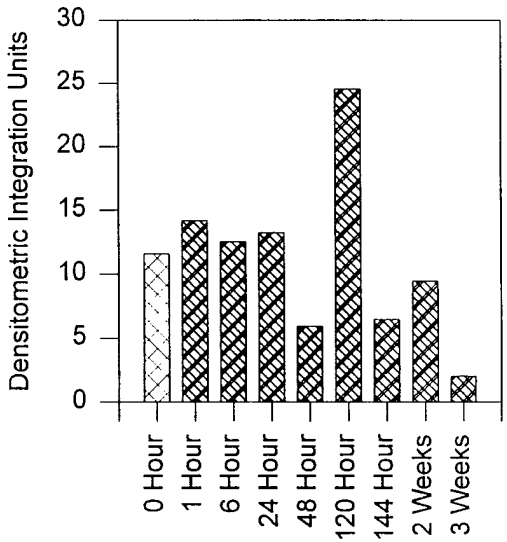


Patient #24: Protein Kinase C Levels vs. Treatment with Bryostatin-1

PKC-alpha



PKC-delta



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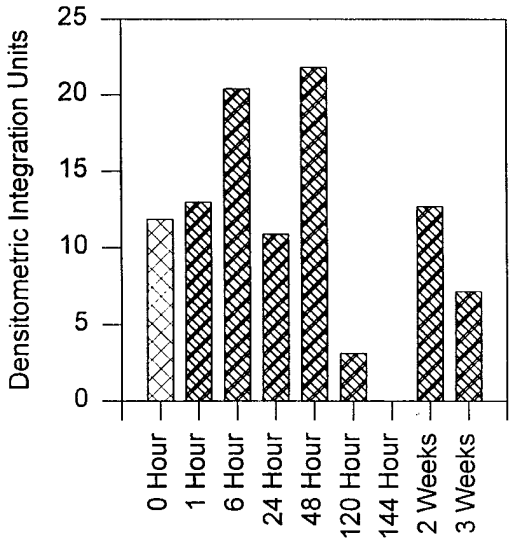
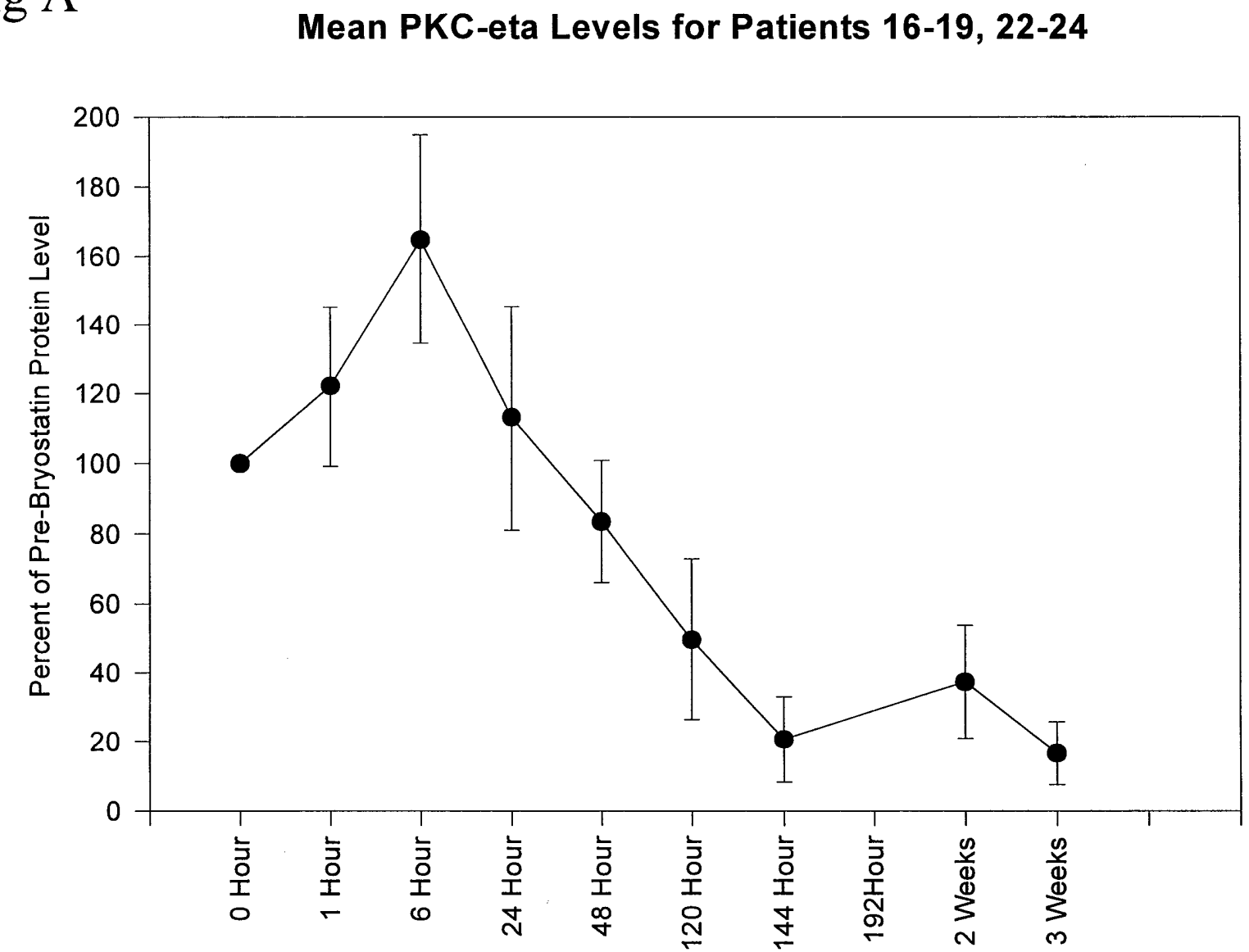


Fig A


Mean PKC-eta Levels for Patients 16-19, 22-24
Percent of Pre-Bryostatin Protein Level
200
180
160
140
120
100
80
60
40
20
0
0 Hour
1 Hour
6 Hour
24 Hour
48 Hour
120 Hour
144 Hour
192Hour
2 Weeks
3 Weeks

B) MMP-9 assays

The expression of the matrix metallo-proteinase MMP-9 has been shown by us and others to be highly regulated by agents that alter PKC activity and levels in cells. This effect is mediated at the level of gene expression; the promotor of the gene containing PKC responsive elements. MMP-9 and MMP-2 (a non-PKC responsive enzyme) are easily assayed in patient plasma using a technique called gelatin zymography. For these reasons it was decided to study MMP 9 and 2 levels in patient plasma with the idea that MMP-9 levels might be modulated by the drug and provide an indication of the level of suppression of PKC activity.

Briefly, patient blood is collected in vacuum tubes containing EDTA as an anticoagulant. The whole blood is then centrifuged to remove the blood cells and the plasma carefully removed, aliquoted in tubes and frozen at -70C until assayed. Zymography is conducted by standard methods on diluted plasma. Patient samples are diluted 1:10 with water and a 10 µl sample of each time point is further diluted 1:1 with sample buffer and run on 7% poly acrylamide gels that contain 0.1% gelatin. After running, the gels are incubated twice for 30 minutes in 2.5% Triton X-100 to remove the SDS and then incubated overnight at 37°C in 5 mM $CaCl_2$, 1% Triton X-100, 50 mM tris pH 7.4 to allow digestion of the gelatin by the MMPs in the samples. Gels are subsequently stained with Coomassie Brilliant Blue to allow areas of digestion to be visualized as areas of clearing in a blue background. Conditioned medium from HS578t cells that have been treated with APMA (4-aminophenylmercuric acetate) to cause partial activation of the MMP-9 are also run on the gels as a marker for active and latent MMP-9. The gels are dried between sheets of cellophane and the areas of clearing measured by densitometry. This process gives an indication of the relative levels of activity, though the numbers generated do not have a liner relationship to the amount of enzyme present because the substrate is, by definition, not present in excess.

A graphical representation of the MMP-9 assays is presented on the next pages for the patients that we have completed. MMP-2 data is not shown because the levels of this enzyme were found to be essentially not different between the different time points. This gives a good indication that equivalent amounts of sample were loaded in each lane of the gels. This is reassuring in the face of the tremendous amount of variation that is seen in the MMP-9 levels over quite short time periods. This is an interesting result as this kind of variation has not been reported before. We are in the process of repeating the MMP-9 assays in plasma samples drawn from normal volunteers to determine if this level of variation is usual, and to try and determine if there are any temporal patterns to the changes in level seen. Another interesting finding is that all of the MMP-9 in the patient samples is in the latent, non-active form. This is somewhat surprising as others have seen active enzyme in patient plasma and have suggested that there may be some prognostic or diagnostic significance to the level of activation of the enzyme. We attribute the low level of enzyme activation that we see to the great care that we take to process and freeze the patient samples as soon as they are drawn. We are in the process of exploring this issue further and intend to report our findings soon.

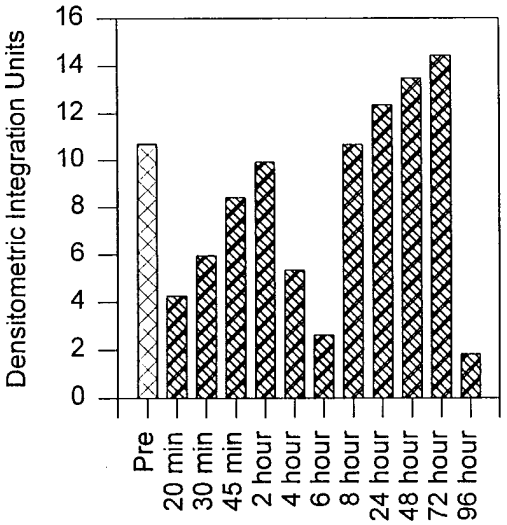
C) Modulation of cell signaling

PKC is known to play an important role in modulating the activity of many if not all of the signaling pathways in the cell. We have, therefore, been testing methods that we can use to assess the level of activity of these pathways with the idea that we can use these methods to measure the effect of Bryostatin-1 on these PKC mediated effects *in vivo*. To do this we have been conducting studies *in vitro*, to identify key regulatory molecules that are modulated by treatment with Bryostatin-1. The results of some of these experiments are detailed below. MCF-7 cells were treated in T25 flasks with 10 nM Bryostatin for the indicated time in their usual growth medium (DMEM, 10% FCS). After this period the cells were washed with PBS and lysed by the addition of boiling lysis buffer (10 mM Tris pH 8, 1% SDS). The samples were then boiled for 5 minutes, sonicated and a small sample was removed to determine the protein concentration. The remainder of the sample was mixed 1:1 with sample buffer and frozen until required at -70C. These samples were then used in a series of western blot experiments to determine the effect of Bryostatin-1 treatment on the condition of the cells and the level and activation state of various proteins.

Bryostatin-1 is known to induce apoptosis in some systems and so the samples were probed with an antibody against the enzyme Poly ADP Ribose Polymerase (PARP), the cleavage of which by prICE and CPP32 is know to be part of the apoptotic cascade. Detection of the cleavage products of this reaction is a standard way of determining the level of apoptosis in cell systems. Blots were analyzed with a rabbit poly clonal-anti PARP antibody and the results are shown in Fig B1. Long-term exposure to Bryostatin-1 clearly causes apoptosis in this system and this may be a useful assay to use on the PBL lysates. We are in the process of assessing this. An important protein in the regulation of apoptosis is anti-apoptotic protein Bcl-2. We studied the level of Bcl-2 in the same samples that were assayed for PARP cleavage. Western blots were analyzed with an antibody that recognizes Bcl-2 and the results are shown in Fig B2. As can be seen after approximately 24 hours exposure there is a slight loss of Bcl-2 protein that becomes more pronounced with further treatment. This is consistent with the subsequent appearance of PARP cleavage. Following the loss of Bcl-2 may be another useful marker of Bryostatin-1 action. As described above, PKC is known to interact with many of the normal signaling pathways in the cell. A key signaling molecule is c-Raf and experiments were conducted to determine if treatment with Bryostatin-1 produced any alteration in this protein. These results are presented in Fig B3. Exposure to Bryostatin-1 results in a very rapid upward motility shift in the Raf-1 protein. This alteration appears to persist at least for the first 48 hours of exposure to the drug. This mobility shift is consistent with the protein becoming phosphorylated and it is known that certain phosphorylation events lead to the activation of this protein. Further work is in progress to determine what these data mean. Raf-1 is responsible for activating the MAP kinase cascade and so we went on to investigate the activity of MAPK in cells treated with Bryostatin-1. As can be seen from Fig B4 there is an upward mobility shift in MAPK very rapidly after treatment with Bryostatin-1. This mobility shift is consistent with the activation of this protein and with the idea that the mobility shift in Raf described above does indeed represent an activation event and that this protein is activating MAPK. We are in the process of evaluating these markers for use in determining the level of Bryostatin activity in the patient samples.

MMP Activity of 92KDa vs. Treatment with Bryostatin-1

Patient #1



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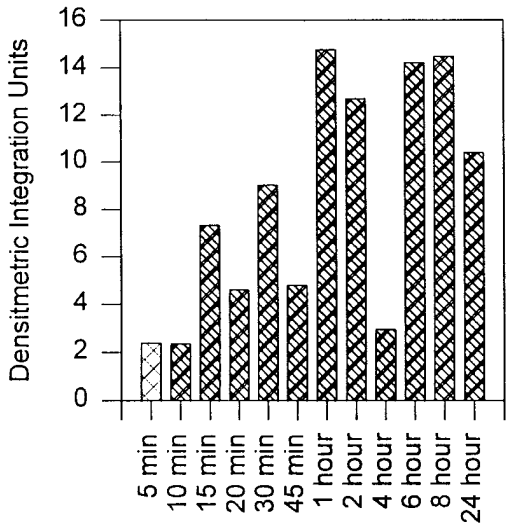

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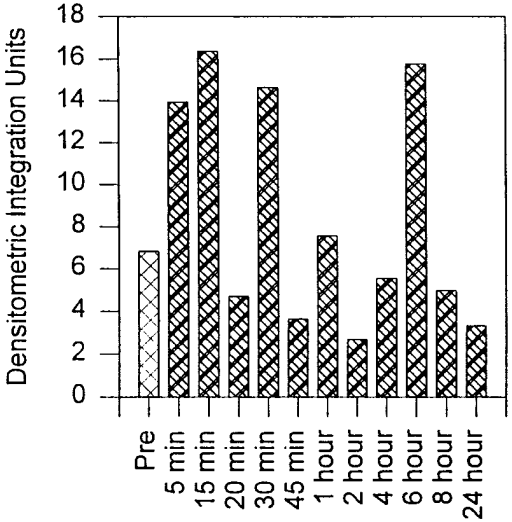

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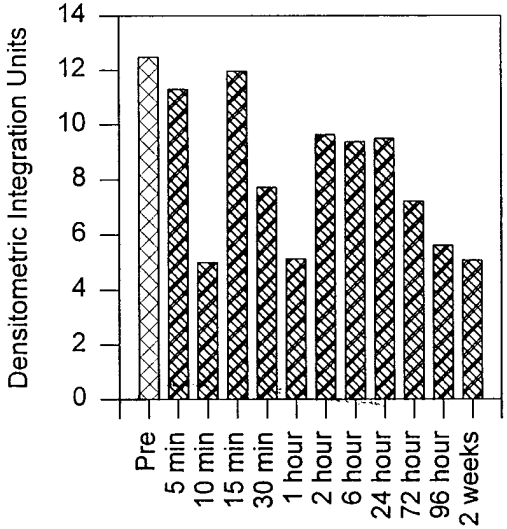

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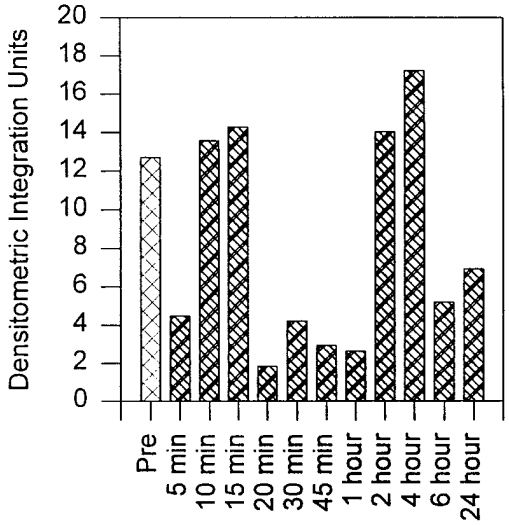

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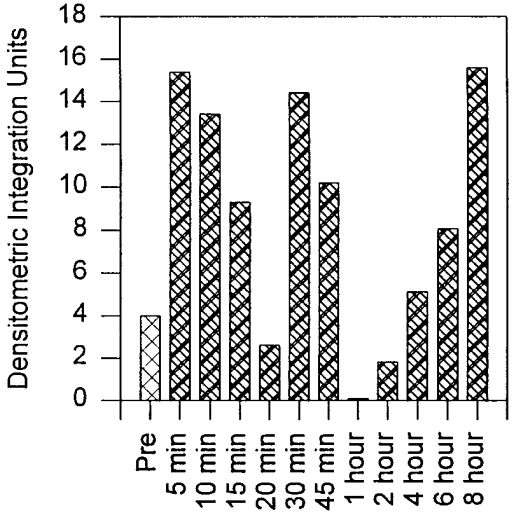
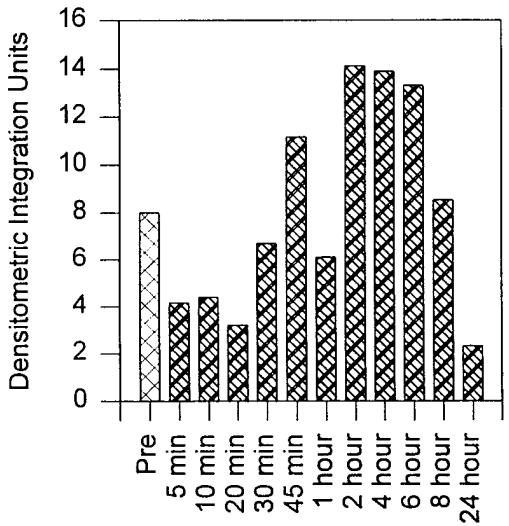


MMP Activity of 92KDa vs. Treatment with Bryostatin-1

Patient #10



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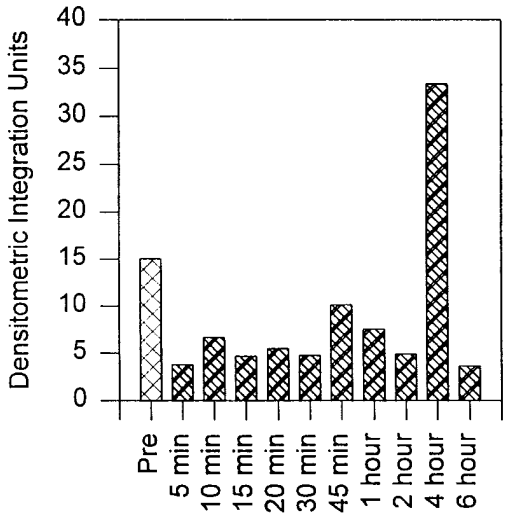

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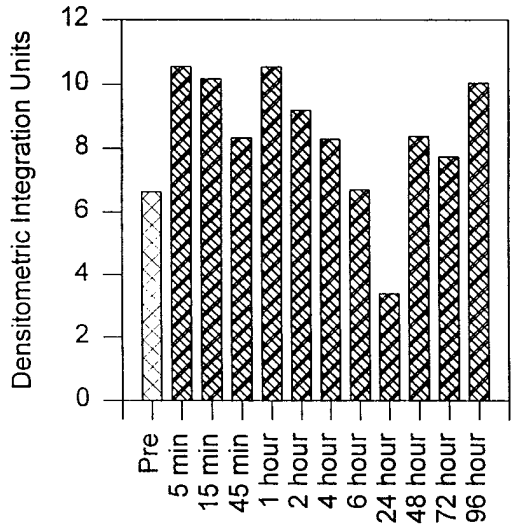

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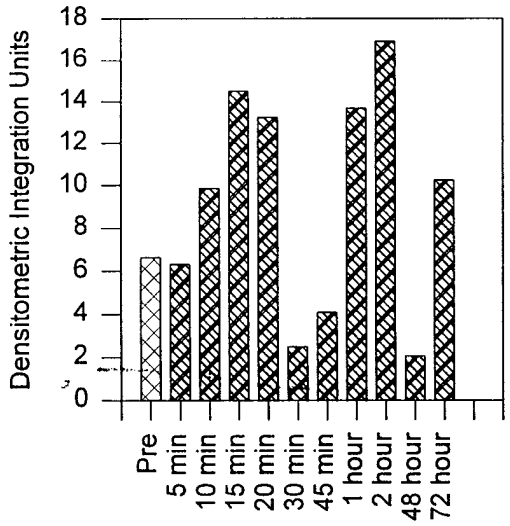

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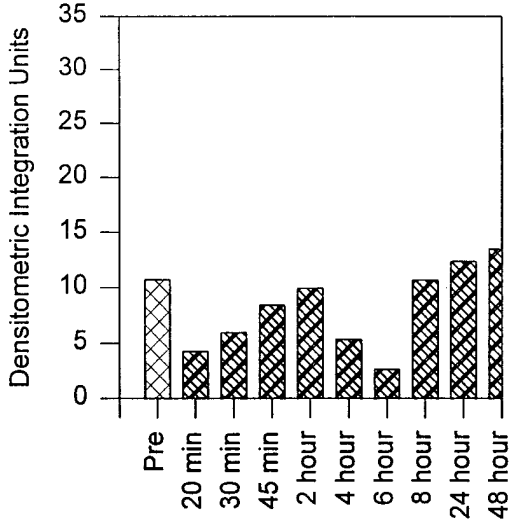

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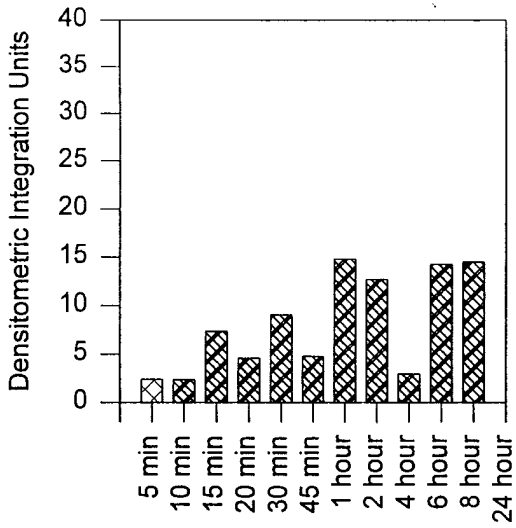
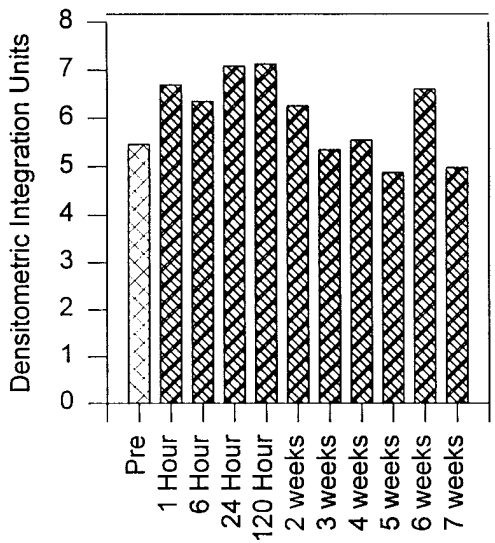


MMP Activity of 92 KDa vs. Treatment with Bryostatin-1

Patient #17



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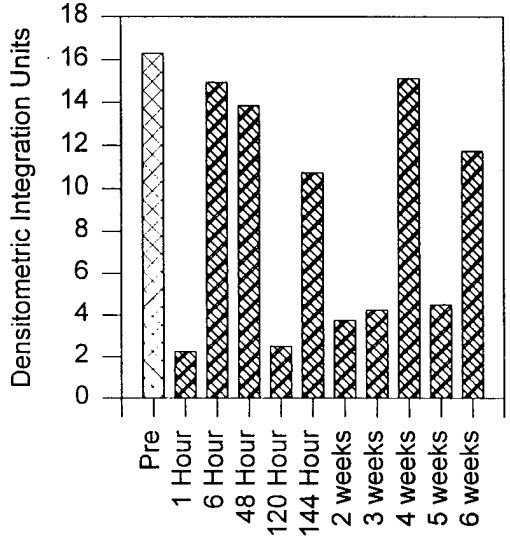

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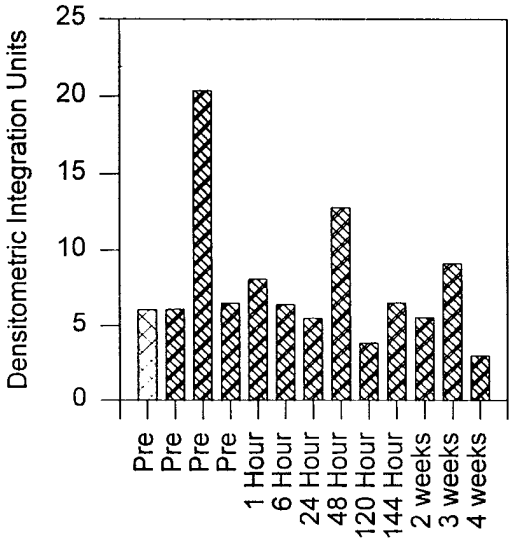

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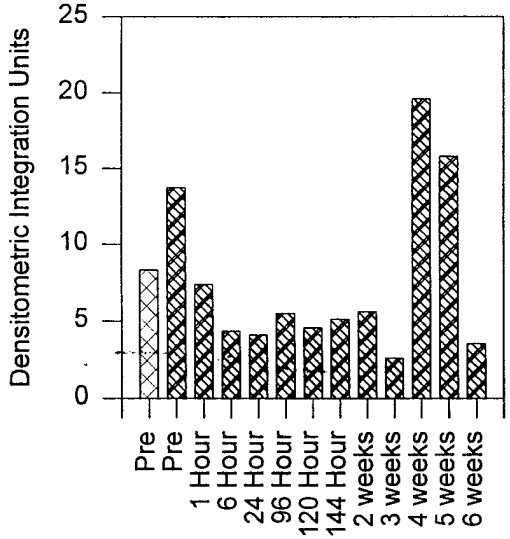

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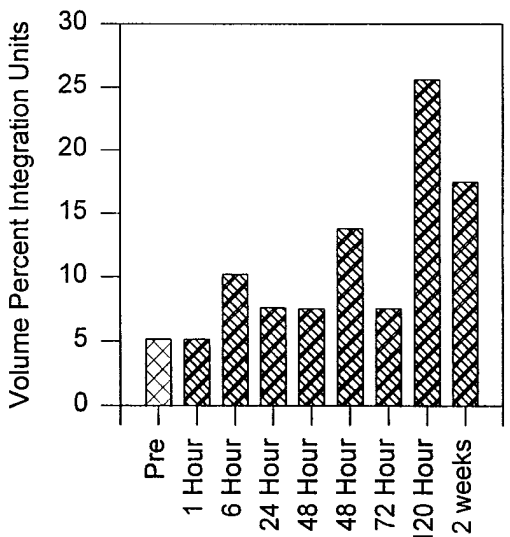


Task 2

Effect of anti-PKC-alpha therapy on PKC levels and PKC signaling pathways in human tumors

It has been shown by a number of research groups that altering the expression of a particular isoform of PKC in a cell causes alterations in the expressions of other forms. However, these data relate largely to transfection experiments, and so are the result of relatively long-term selections. It is, therefore, not clear if these changes are the result of selection for sub-populations of cells that happen to express the altered complement of isoforms, or if indeed there is a direct cause and effect relationship between the increase in one isoform and the suppression of another. This is one of the questions that we hope will be answered by this study. As part of a clinical trial in which patients with accessible metastatic deposits of their tumor will be treated with a specific PKC-alpha molecule (ISIS 3521), we have been obtaining tumor biopsies before and during therapy. These samples have been fixed in formalin and embedded in paraffin. We have shown that we can obtain at least 50 5 micron sections from each of these biopsies and we are preparing to assay these samples for a number of markers to determine the impact of this therapy. We have now collected and processed biopsies from 6 patients and are about to start conducting the analyses described below.

Drug distribution

The distribution of the antisence drug within the tumor biopsy will be analyzed by staining sections with an antibody against the backbone of the drug. This will allow us to map any changes that we see in the tumor tissue to the presence and absence of drug. This will allow us to identify which changes relate to the presence of the drug and which are secondary to other effects such and alterations in the perfusion of the tumor caused by suppression of the blood supply produced by the treatment.

Proliferation and Death

The proliferative activity of the tumor tissue will be assessed before and during therapy with ISIS 3521. Sections of tumor tissue will be stained with an antibody against the proliferation associated nuclear antigen Ki67. We have established the conditions required to stain with this antibody (Novocastra, Newcastle UK), and have started to stain the samples from this trial. The level of apoptosis in the tumor sections will also be analyzed. This will be done with a method with which we have considerable experience - the TUNEL assay. In this method the ends of broken strands of DNA are labeled enzymatically with biotin tagged nucleotides. These biotin tags are then detected using standard methods. Brown staining cells are then examined under the microscope to determine if they have the typical morphologic features of an apoptotic cell (necrotic cells also stain by this method). The number and distribution of apoptotic cells is noted. We will shortly start analyzing the tumor sections from the ISIS trial with this method.

PKC levels

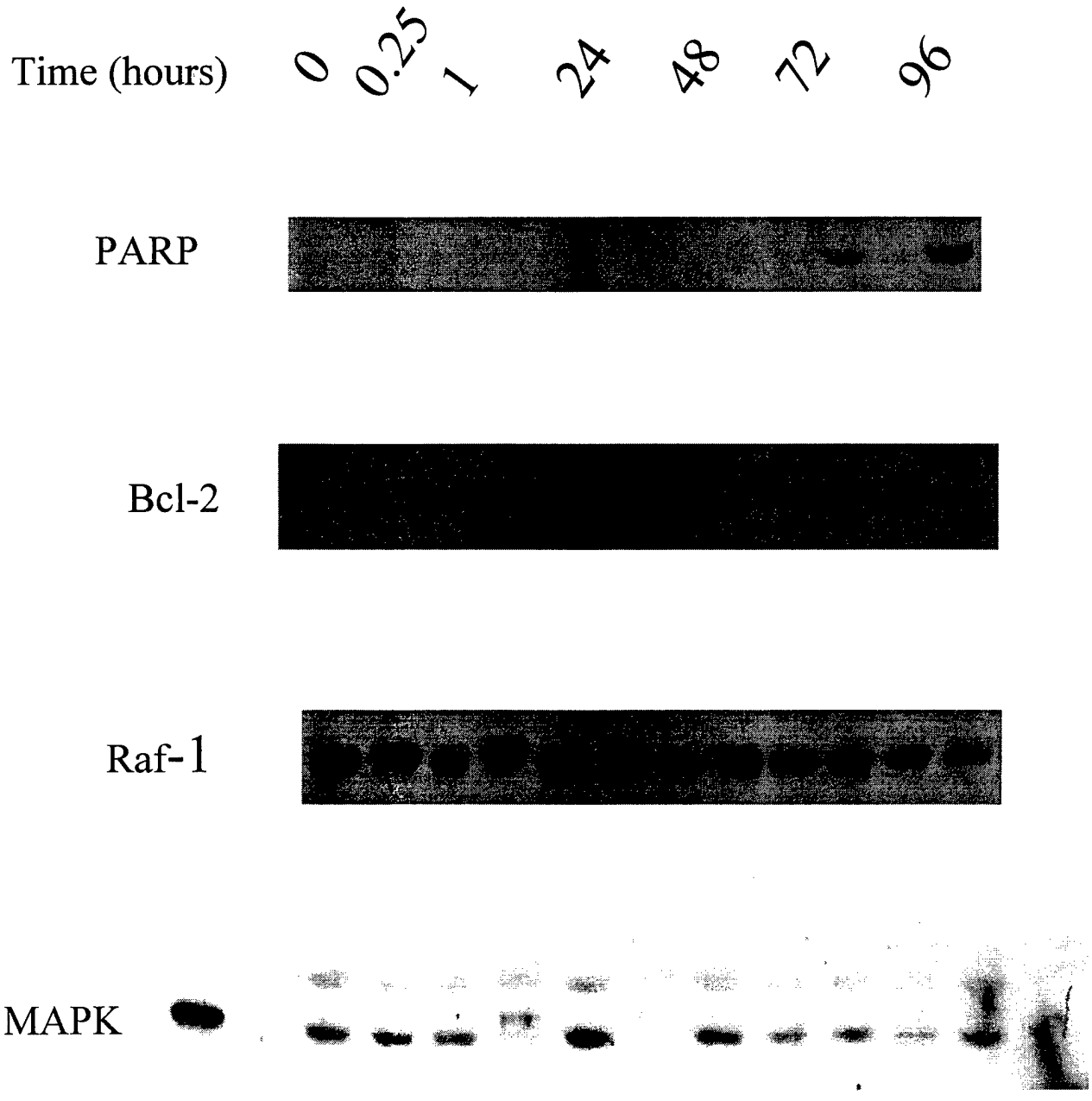
We would expect that therapy with the anti-PKC-alpha antisence molecule would result in the

suppression of PKC-alpha levels and we will examine this by immunohistochemistry (IHC) for the protein in the sections. However, as noted above, there is reason to believe that other isoforms of the enzyme might be effected and so we are in the process of establishing IHC assays for other isoforms.

Depending on the results that we obtain, we will also stain sections with antibodies against other proteins and markers of signaling activity. For example, if we see a significant increase in the level of apoptosis in the tumor sections that are being treated with the compound then we would stain the sections with antibodies against the proteins that are known to mediate apoptotic signals - the Bcl family members.

We believe that this unique study will provide us with invaluable insights into the function of PKC in the regulation of metastatic cancer.

Fig B. Effects of Bryostatin-1 on PARP cleavage BCL-2 protein levels, Raf and MAPK phosphorylation.



Task 3

Direct measurement of PKC kinase activity

One of the objectives of the original proposal was to develop a method to allow the level of PKC activity to be determined in cells and tumors. All of the existing assays were in reality assays of PKC levels rather than activity, and many could not be used *in vivo*. Our approach was to generate a reporter construct in which PKC activity could be estimated from the signal produced by a reporter enzyme, the expression of which was being driven by a PKC responsive promotor. As reported in our annual reports, we have generated the construct and it appears to work well. However, as the literature on signal transduction pathways has grown, it has become increasingly clear that there is no such thing as a "pure' PKC response element. The element that we used to generate our construct is still the best approximation to that goal, but it is clear that signals to a cell that are not being directly mediated through PKC activity also have the potential to alter the output from our reporter construct. For this reason we have developed a parallel strategy that will provide a much more specific indication of the level of PKC kinase activity both *in vitro* and *in vivo*. A relatively new reporter molecule that is being increasingly used in a variety of studies is the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria.* A modified form of this protein has been generated that has altered excitation and emission spectra so that it works well with detection systems optimized for fluorescein.

We have now developed two chimeric GFP molecules each of which contain as a peptide fused to the c-terminus of the GFP molecule a substrate site for phosphorylation by PKC-alpha. The structure of these two molecules is shown in Fig C. We expect that the protein will be synthesized in the cells and that a portion of the chimeric molecules will then become phosphorylated by PKC-alpha. The proportion of the protein that is phosphorylated should provide an indication of the level of PKC activity in that cell. To determine the ratio of native versus phosphorylated protein, we will make use of the innate fluorescence of the molecule and a high performance separation technique - capillary electrophoresis (CE). Crude, whole cell lysates from cells transfected with the constructs will be prepared and run over the capillary electrophoresis column under conditions that will resolve the native and phosphorylated form of the chimeric GFP molecule. The level of PKC activity should be proportional to the size of the phosphorylated GFP peak. This technique will also be applicable to cells grown as xenografts in nude mice.

We have done transient transfection experiments with these two molecules and have shown that the additional amino acids have not quenched the fluorescence of the GFP. We are in the process of developing the capillary electrophoresis assay that we will use to resolve native from the phosphorylated forms of the molecules. Promising preliminary data are presented in Fig D. This shows a trace from the CE assay indicating that there are indeed two separable forms of the protein within the cells.

Fig C

GFP

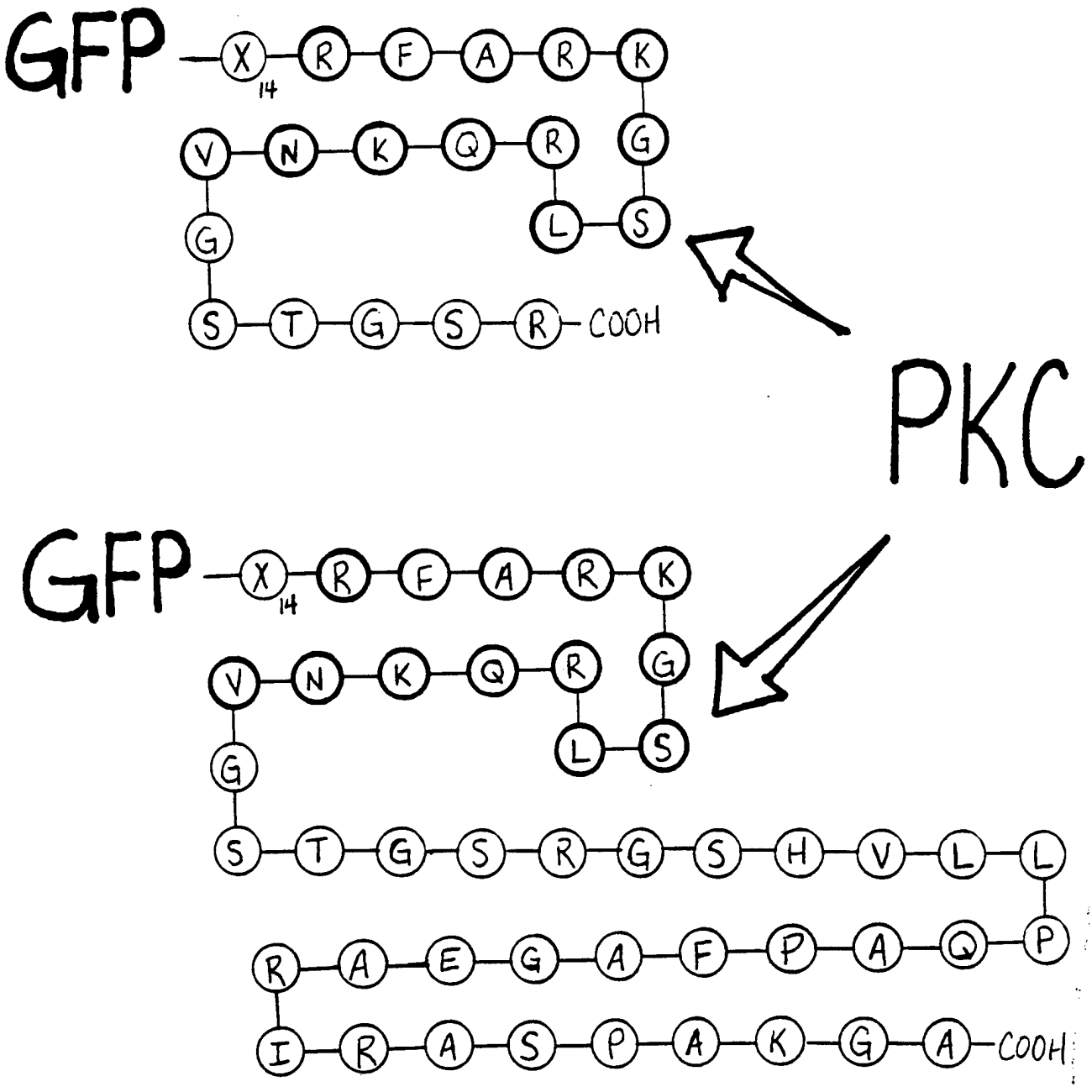
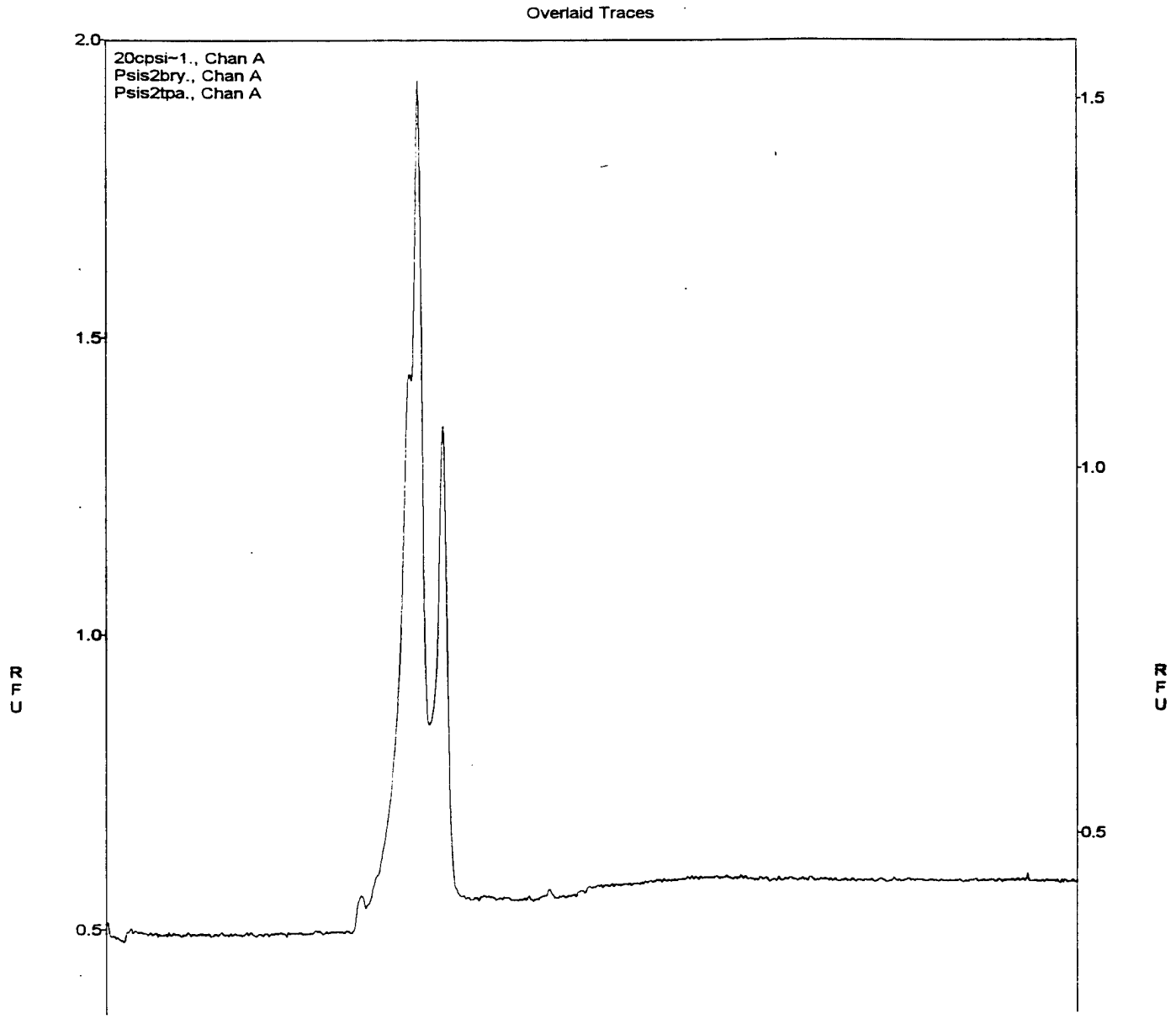

GFP
X14 R F A R K
V N K Q R G
L S
G
S T G S R COOH
PKC
GFP
X14 R F A R K
V N K Q R G
L S
G
S T G S R G S H V L L
R A E G A F P A Q P
I R A S P A K G A COOH

Fig D


Overlaid Traces
20cpsi~1., Chan A
Psis2bry., Chan A
Psis2tpa., Chan A
2.0
1.5
1.0
0.5
RFU
1.5
1.0
0.5
RFU

Conclusions:

In summary: we have made significant progress towards the completion of the modified technical objectives of the proposal. We believe that these studies will considerably enhance our understanding of the importance of PKC as a target for the therapy of metastatic cancer. The approaches that we are using take advantage of unique opportunities to conduct the studies using clinically relevant samples. This makes the work inherently more difficult, but also enhances that potential pay-off.

Abbreviations

APMA	4-Aminophenylmercuric Acetate
C-Terminus	Carboxy-terminus
CE	Capillary Electrophoresis
DMEM	Dulbeco's Modified Eagles Medium
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
GFP	Green Fluorescent Protein
IHC	Immunohistochemistry
IRB	Institutional Review Board
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metallo Proteinase
PBL	Peripheral Blood Lymphocytes
PKC	Protein Kinase C
SDS	Sodium Dodecile Sulphate

Appendix A

AMENDMENT TO THE TECHNICAL OBJECTIVES OF DAMD 17-94-J-4166.

Michael D. Johnson, Ph.D.

The purpose of this document is to request approval for a shift in the emphasis of the Technical Objectives of the Army Career Development Award to Michael D. Johnson, Ph.D. - DAMD 17-94-J-4166. As outlined in our annual reports to date, we have made considerable progress towards completing the technical objectives outlined in the original proposal. However, in the years since the proposal was written, considerable progress has been made in our understanding of the role of PKC in intracellular signaling and in its potential role in disease. Furthermore, drugs that could only be used in animal model systems at that time, have now made there way into experimental clinical medicine.

In the light of these advances some of the experiments proposed in the original submission have become less interesting, in part because the questions they were designed to answer have be answered by other experimental approaches, and partly, because there are now better ways to answer some of those questions. To take advantage of these advances in the field, we would like to alter the direction of this grant as detailed below. The proposed changes do not materially alter the thrust of the proposal, but we believe will provide more relevant information, will avoid needlessly duplicating the work of others and will take advantage of several unique opportunities that have presented themselves. Attached at the end of this document is a modified Statement of Work that incorporates the changes proposed.

1) Effect of Bryostatin-1 on PKC isoform levels and activity *in vivo.*

Bryostatin-1, is a macro-cyclic lactone that has been shown to inhibit the activity of PKC in a number of experimental systems. In the original proposal, we were going to conduct a number of experiments to determine the effects of this drug on PKC activity in the nude mouse. However, this compound is now being used in experimental clinical medicine and so an ongoing clinical trial with this agent at the Cancer Center has provided the opportunity to do a much more incisive and clinically relevant study. With IRB approval we have been collecting a variety of samples from patients that are receiving Bryostatin-1 through this clinical trial and the analysis of these samples will allow us to determine the effect of this agent on PKC activity and the activity of down-stream signaling pathways.

A) Determination of PKC isoform levels in peripheral blood lymphocytes

We are isolating peripheral blood lymphocytes from these patients and are preparing lysates from the cells for the analysis of PKC levels. The blood is clearly not the most relevant tissue in these patients, but has the advantage that we can obtain sequential specimens with minimal discomfort

to the patients and furthermore, blood cells express almost all isoforms of PKC. This allows the analysis of the impact of treatment with Bryostatin-1 on all of the isoforms to be conducted in parallel. In a number of cases we have been able to biopsy the tumors of patients both before and during therapy, and these tissues will allow us to determine how reliable the blood cells are as a model for the action of Bryostatin-1 in tumor tissue. This issue will be addressed in greater detail in a subsequent study described below.

B) Plasma MMP-9 levels

We have previously shown that the synthesis of the matrix-metallo-proteinase MMP-9 can be modulated by treatment with PKC activators and inhibitors. Using a simple assay we can measure the level of MMP-9 in the plasma of patients receiving Bryostatin-1. We are collecting plasma from these patients prior to, during and after Bryostatin-1 infusion and are in the process of analyzing the MMP-9 levels in these samples.

C) Modulation of cell signaling

Since the original proposal was written, a series of new reagents have become available that make possible a series of additional studies that can be conducted on the samples described above. PKC activity has been shown to impinge on the activity of a series of down-stream signaling pathways within the cell. These include the p44/42 MAP kinase signaling cascade, the p38 MAP kinase pathway, the JNK kinase pathway and the JAK/STAT pathway. A series of antibodies to key regulatory molecules on all of these pathways have been developed which the activation state of the proteins to be determined. This allows the activity of a particular pathway to be assessed by western blot analysis. We propose to use these reagents to determine the effect of the modulation of PKC activity by Bryostatin-1 *in vivo* on the activity of these signaling pathways know to be impacted by PKC. This will provide valuable information as it is not known what pathways are most important in the mediation of PKC directed signaling in this context, and more particularly, which pathway will be most significantly inhibited by Bryostatin-1. To compliment these experiments we will extend some existing *in vitro* studies into this clinical system. We have also demonstrated *in vitro* that we can follow specific phosphorylation events caused by transitory activation of PKC by Bryostatin-1. This transitory activation is thought to be an important part of the mechanism by which Bryostatin-1 ultimately results in PKC inhibition. We will attempt to repeat these studies using the patient samples. The protein c-raf is an example of a peptide that is rapidly phosphorylated on treatment with Bryostatin-1 and this phosphorylation event can easily be followed by western blot analysis due to the molecular weight shift that it produces. We will investigate this effect in the patient samples to determine if similar early activation events are seen *in vivo*.

2) Effect of anti-PKC-alpha therapy on PKC levels and PKC signaling pathways in human tumors.

Our work and that of others has shown that altering the level of one isoform of PKC in a cell line causes the levels of the other isoforms expressed in that cell to change. A new clinical trial to be conducted here at Georgetown will allow a unique opportunity to determine if this happens *in vivo* in tumor tissue, and what impact these changes may have on cell signaling pathways and on the proliferative and apoptotic status of the tumor. ISIS 3521 is a modified antisence drug directed against PKC-alpha. It has been shown to cause down-regulation of PKC-alpha levels in cells that take it up and it has been demonstrated in phase I clinical trials that tumor cells take the drug up and show a reduction in PKC-alpha levels. With the approval of the IRB, we will obtain needle biopsies of visceral metastatic deposits of tumors in patients before and during treatment with ISIS 3521. These samples will be fixed and imbedded in paraffin. We have shown that we can get of the order of 50 5 micron sections from each biopsy and these sections will be stained with various antibodies to allow us to answer several important questions.

We will stain sections of each the biopsies with anti-PKC antibodies to determine firstly if PKC-alpha levels are changed by the therapy and then to determine if the level of any of the other isoforms are altered. The drug has been shown in extensive testing to be completely specific for PKC-alpha. We will stain adjacent sections with an antibody that can detect the drug. This will allow us to be sure that the effects seen are the direct result of the drug and not due to some other phenomenon such as alterations in the pO_2 secondary to drug effects on the blood supply to the tumor. We will also try and obtain paraffin blocks of the original tumors that resulted in the metastatic deposits that we are analyzing. This will allow us to compare the characteristics of these tumors to the metastases to determine if there is any pattern in the isoforms of PKC that are expressed and if there is any correlation with the proliferative or apoptotic state of the tumor or metastasis.

Many of the activation state dependent antibodies to key signaling molecules described above work in paraffin fixed tissues and so sections will also be stained with these antibodies to determine if altering PKC-alpha levels has altered signaling down any of these pathways. To asses the proliferative state of the tumor tissue, both before and during treatment, sections will be stained with an antibody that recognizes the proliferation associated nuclear antigen Ki-67. There is some evidence that reductions in the levels of PKC-alpha produced by treatment with ISIS 3521 can result in cells undergoing apoptosis. We will therefore asses the apoptotic rate in the tumor samples both before and during treatment. This we will do by use of the TUNEL assay which stains the nuclei of cells that contain cleaved ends of DNA - a characteristic of apoptosis. Inspection of the cells staining for the characteristic morphology of a cell undergoing apoptosis allows conformation. Even though uptake of the drug into the cells will produce a background - it is effectively a DNA molecule - we have established that we will be able to detect apototic cells in the face of this drug produced background.

These studies should allow us to answer many of the key questions that remain to be answered about the impact of altering the level of one PKC isoform, both on the level of the others and on the down stream signaling pathways. These alterations can then be correlated to effects on the proliferative and apoptotic state of the cells, allowing us to determine the relevance of these

changes to the desired outcome IE preventing the growth of the tumor, or indeed killing the tumor cells.

3) Direct measurement of PKC kinase activity.

One of the objectives of the original proposal was to develop a method to allow the level of PKC activity to be determined in cells and tumors. All of the existing assays were in reality assays of PKC levels rather than activity, and many could not be used *in vivo*. Our approach was to generate a reporter construct in which PKC activity could be estimated from the signal produced by a reporter enzyme, the expression of which was being driven by a PKC responsive promotor. As reported in our annual reports, we have generated the construct and it appears to work well. However, as the literature on signal transduction pathways has grown, it has become increasingly clear that there is no such thing as a "pure' PKC response element. The element that we used to generate our construct is still the best approximation to that goal, but it is clear that signals to a cell that are not being directly mediated through PKC activity also have the potential to alter the output from our reporter construct. For this reason we have developed a parallel strategy that will provide a much more specific indication of the level of PKC kinase activity both *in vitro* and *in vivo*. A relatively new reporter molecule that is being increasingly used in a variety of studies is the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria.* A modified form of this protein has been generated that has altered excitation and emission spectra so that it works well with detection systems optimized for fluorescein. We propose to generate a chimeric GFP expression construct that produces a fusion protein containing a peptide sequence, know to be a substrate for PKC-alpha, fused to the C-terminus of GFP. We will then transfect cells with this construct and select stable transfectants. We expect that the protein will be synthesized in the cells and that a portion of the chimeric molecules will then become phosphorylated by PKC-alpha. The proportion of the protein that is phosphorylated should provide an indication of the level of PKC activity in that cell. To determine the ratio of native versus phosphorylated protein, we will make use of the innate fluorescence of the molecule and a high performance separation technique - capillary electrophoresis (CE). Crude, whole cell lysates will be prepared and run over the capillary electrophoresis column under conditions that will resolve the native and phosphorylated form of the chimeric GFP molecule. The level of PKC activity should be proportional to the size of the phosphorylated GFP peak. This technique will also be applicable to cells grown as xenografts in nude mice.

Other isoforms of PKC can also phosphorylate this peptide sequence, and so the level of phosphorylated GFP will provide an indication of the aggregate PKC activity in the cell. We will do a series of experiments to validate the construct and to determine the relationship of the signal that is produced to the level of signal from our PKC responsive reporter construct. Stably transfected cells will be treated with agents that are known to active or inhibit the activation of PKC, and the phosphorylation state of the GFP within the cells determined by CE. After the correct function of the construct has been established and the output of the assay calibrated against the other measures of PKC activity, we will proceed with the experiments outlined in the

original proposal.

Summary:

The changes in the direction of studies to be carried out for the grant DAMD 17-94-J-4166 outlined here, capitalize on the significant advances in our understanding of PKC signaling and realign the proposal to take advantage of anti-PKC directed clinical trials being conducted at this institution. We believe that the result is a stronger more relevant series of experiments that will generate data that will be extremely useful in furthering our understanding of the effects if anti-PKC therapy, and indeed the importance of altered PKC activity in the pathology of breast cancer.

STATEMENT OF WORK (SOW):

The amended SOW presented below indicates how the work described above will be conducted in the 18 months remaining in DAMD 17-94-J-4166.

Task 1: Effect of Bryostatin-1 on PKC isoform levels and activity *in vivo*.
Months 1-18

-Collect PBL samples from patients receiving Bryostatin-1
-Collect plasma samples from patients receiving Bryostatin-1
-Analyze PKC levels in PBL lysates
-Analyze MMP-9 levels in patient plasma
-Evaluate other markers of Bryostatin-1 action
-Analyze patient samples for changes in signaling pathway activity

Task 2: Effect of anti-PKC-alpha therapy on PKC levels and PKC signaling pathways in human tumors. **Months 10-18**

-Collect biopsies from patients prior to and during therapy with ISIS 3521
-Analyze PKC levels in biopsies
-Measure proliferative state of biopsy tissue
-Determine level of apoptosis in tumor biopsies
-Establish activation state of cell signaling pathways.
-Analyze distribution of drug in samples from treated patients.

Task 3: Direct measurement of PKC kinase activity. **Months 1-18**

-Generate chimeric GFP reporter molecule
-Construct reporter plasmid
-Verify activity of construct
-Develop CE assay
-Generate stable transfectants with GFP construct
-Determine effect of PKC activators and antagonists on phosphorylation state of GFP construct
-Conduct assays to determine effect of the extracellular environment on PKC activity
-Determine PKC activity level in cells grown in the nude mouse.

Abbreviations:

C-Terminus	Carboxy-terminus
CE	Capillary Electrophoresis
GFP	Green Fluorescent Protein
IRB	Institutional Review Board
MMP	Matrix Metallo Proteinase
PBL	Peripheral Blood Lymphocytes
PKC	Protein Kinase C