ANION-EXCHANGE CHROMATOGRAPHY OF THE MURINE ESTROGEN RECEPTOR: IONIC FORMS OF RECEPTOR UNDER DIFFERENT STATES OF ACTIVATION

A Thesis

Presented to

the Faculty of the College of Arts and Sciences

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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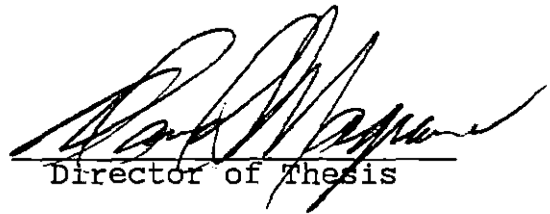
Accepted by the faculty of the College of Arts and Sciences, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.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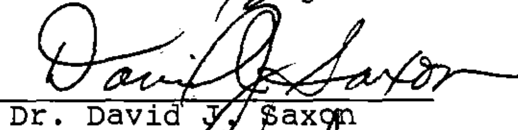
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ABSTRACT

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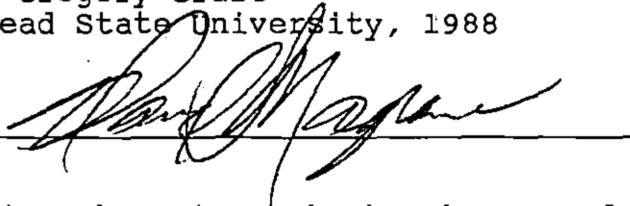
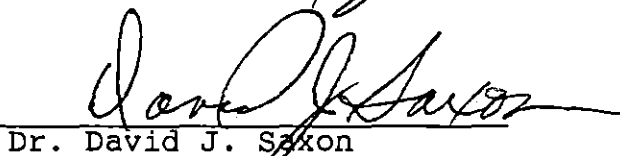
Gregory Craft
Morehead State University, 1988

Director of Thesis: 

Size-exclusion chromatography has been used in clinical laboratories to develop a "receptor profile" for patients undergoing endocrine therapies. Recently, the need has arisen to develop an "ionic profile" of the estrogen receptor so that more precise assignments can be made concerning endocrine therapies. In this work, a DEAE-cellulose chromatography column was used to separate the ionic forms of the murine estrogen receptor by high performance ion-exchange chromatography (HPIEC). Cytosols from murine uteri were incubated for 1-2 hours with [^{3}H]17B-estradiol, cleared of unbound ligand, and applied to an anion-exchange column. Components were eluted at pH 7.4 using a gradient of phosphate buffer and sodium chloride buffer at 0-4 degrees Celsius. Each salt buffer system eluted the various isoforms

differently, however, both systems eluted peaks in the 0-400 mM range of the gradient. Four isoforms were found with each buffer system. Also, each isoform could be found to elute at nearly the same fraction number under different states of activation, which demonstrates that the isoforms are stable under different incubation conditions. In general, the results obtained show fair reproducibility, good recovery, and demonstrates that high performance ion-exchange liquid chromatography has an apparent role in the purification of proteins where degradation is a problem.

Accepted by: ,Chairman
Dr. David T. Magrane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ACKNOWLEDGEMENTS

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INTRODUCTION

Estrogenic steroid hormones have many sites of action including the uterus, hypothalamus, liver, and kidney [1]. They exert their effects by passing through the cell membrane, interacting with a cytoplasmic binding protein, and, as an activated binding complex, translocating to the nucleus where interaction with chromatin presumably initiates certain events responsible for alterations in the rates of transcription, translation, and even replication [2].

The estrogen binding protein content of human breast cancers has been used to predict the responsiveness of patients to endocrine therapy [2,4-6]. According to Wittliff _et al_ [3], 55% to 65% of primary breast tumors and 45% to 55% of metastatic breast tumors contain more than 10 fmol/mg cytosol protein of estrogen receptors. In this work, the estrogen binding protein is referred to as the estrogen "receptor" and the assays performed on these proteins develop a "receptor profile" for the patient undergoing the endocrine therapy [3].

The estrogen receptor has been studied by several methods such as 1) charcoal-dextran assays, 2) hydroxyl-

apatite method, 3) sucrose gradient sedimentation, and 4) electrophoretic analysis [2,7]. One disadvantage that these methods share is the time involved to run these assays (e.g. some require overnight incubation and/or centrifugation). Proteolysis and many degradative changes can take place while these procedures are being performed.

One method of estrogen receptor analysis that requires little time for preparation and is used extensively is high performance liquid chromatography (HPLC). While there are many types of HPLC, the most widely used has been size-exclusion HPLC. This offers a rapid and reproducible means by which a biopsy sample can be analyzed for "receptor positive" status [8].

Using size-exclusion HPLC, it has been found that the estrogen receptor can exist in different forms, called isoforms, depending on the conditions under which the sample is prepared for HPLC analysis. Using sedimentation analysis, the qualitative relationships between the different receptors of the rat uterus have been determined: cytoplasmic aggregated ~8S, deaggregated ~4S, trypsinized ~3.6S; nuclear ~5S. While the estrogen receptor is mainly referred to as being cytoplasmic, Pavlik _et al_ [9], using DNAase and several protease inhibitors, presented results indicating that a large nuclear receptor exists which displays different

characteristics than the cytoplasmic receptor. This nuclear receptor was isolated from chromatin and, under the conditions used, had a molecular weight greater than 669,000. The intact, non-deaggregated cytoplasmic receptor has a molecular weight of 90-110,000 [9].

"Activation" is a term used in estrogen receptor research to describe certain characteristics of the estrogen receptor in *in vitro* experiments [10]. The "activated" receptor displays a change in ligand dissociation kinetics from fast to slow [10], faster nuclear translocation, and a higher affinity for chromatin [2-5]. Activation can be visualized in a chromatogram by noting a transfer in distribution from one isoform to another (Figure 1). The rat uterine estrogen-receptor complex can be activated *in vitro* by increasing time or temperature of incubation with ligand or by the addition of nucleoside triphosphates [10]. Addition of 10 mM molybdate to cytosol inhibits activation of receptor complexes by some ill-defined mechanism [2-5,10-14]. Since molybdate is a phosphatase inhibitor, activation may involve dephosphorylations [11].

Receptor activation also alters the surface charge of the receptor probably as a result of exposure of charged residues within the oligonucleotide binding site

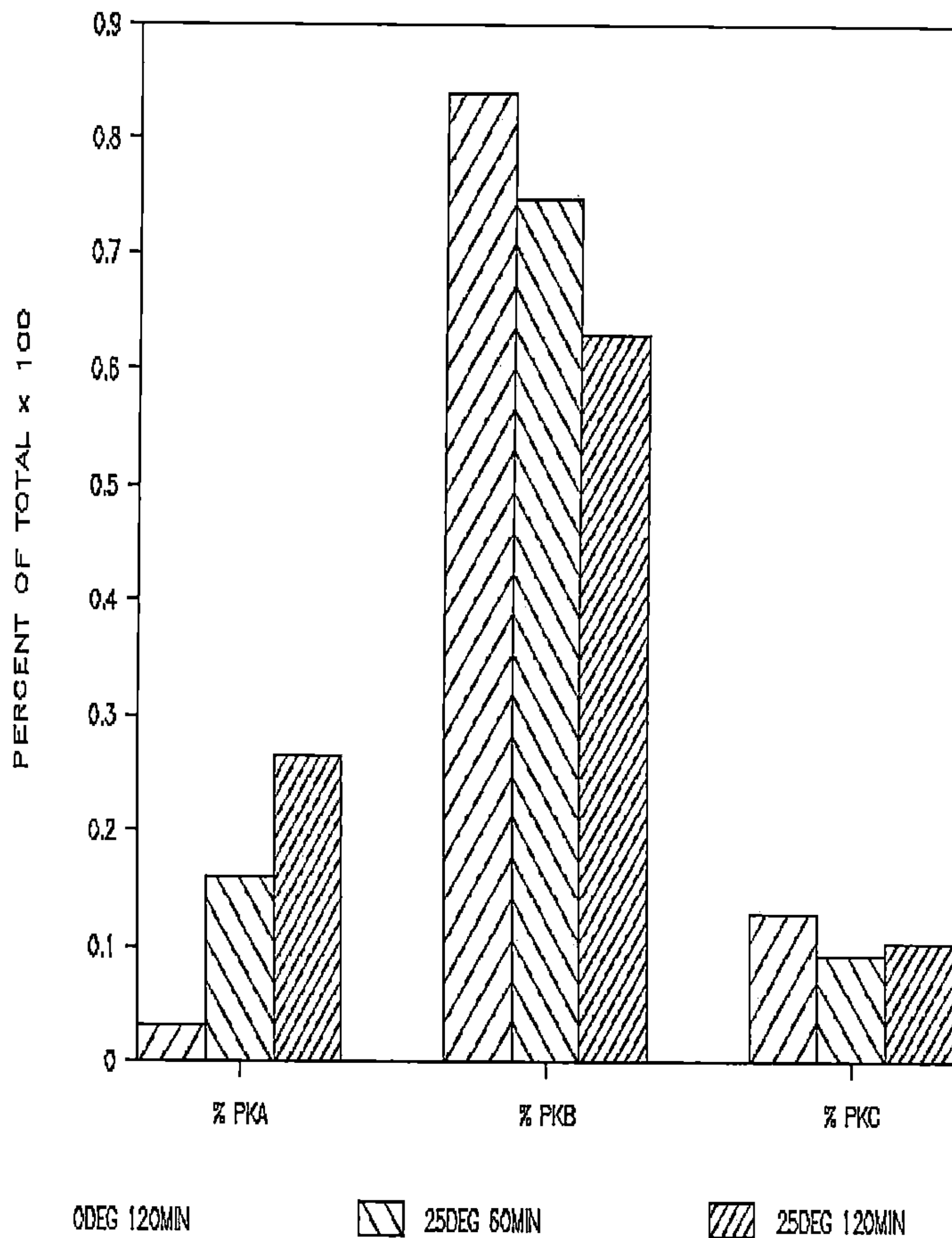

Figure 1. Example showing activation of receptor with size-exclusion chromatography. Activation causes a shift in proportion from Peak B to Peak A.

of receptor [10]. Ion-exchange chromatography is used to investigate the ionic characteristics of the estrogen receptor. While most HPLC buffer systems used in estrogen receptor research favor a neutral pH (7.0-7.4), high salts, and the addition of dimethylformamide, ion-exchange buffer systems also utilize a salt concentration gradient in the system which allows the visualization of different ionic forms of the receptor. By developing an "ionic profile" of the estrogen receptor, more precise assignments can be made concerning endocrine therapies.

Materials and Methods

Materials

Radiolabeled steroid, 17B-[2,4,6,7-$^{3}H_4$]estradiol (104-115 Ci/mmol), was obtained from New England Nuclear, Boston, MA 02118. The following biochemicals were used: sodium acetate, potassium phosphate monobasic, potassium phosphate dibasic, tris acetate, dextran C (Fisher Scientific, Fair Lawn, NJ 07410). Sodium molybdate (Mallinckrodt, Co.,St. Louis, MO 63134), tris, ultra pure grade (Schwarz/Mann,Cleveland, OH 44128), dimethyl-formamide (Burdick & Jackson Chemicals, Muskegon, MI. 49442), ethylenediamine-tetraacetic acid, sodium azide, and sodium chloride were from Sigma Chemical Co., St. Louis, MO 63178. Protease inhibitors, phenylmethyl sulfonyl fluoride, pepstatin A, aprotinin, leupeptin, and ethylenediamine-tetraacetic acid (EDTA) were from Boehringer Mannhein Biochemicals, Indianapolis, Ind. 46250. Immature CF-1 mice (Harlan-Sprague-Dawley, Indianapolis, IN 46229) were obtained at 19-23 days of age and killed within seven days. Millex GV filters (25 mm in diameter, 0.22 um pore size) were from Millipore Corp., Bedford, MA 01730.

Buffers

The concentration of K_2HPO_4 was varied between 1000 mmol/L (P_{1000}) and 10 mmol/L (P_{10}), but the pH was kept at 7.0. All elution buffers contained 75 ml of dimethylformamide per liter. All buffers were membrane filtered with Millipore HATF ultrafiltration membranes to remove particulates.

Preparation of Cytosols

Mice were sacrificed by cervical dislocation and decapitation to bleed out any blood-borne hormones. Freshly removed uteri, freed of fat and mesenteric tissue, were homogenized with a Brinkman Polytron Homogenizer in buffer according to protocol (five uteri per milliliter of buffer)(Table 1). After centrifugation at 3200 x g on a Beckman J6-B ICL centrifuge at 0 degrees Celcius for 30 minutes to remove the nuclear-myofibrillar material, the supernatant was recentrifuged on a Beckman L8-70 Ultracentrifuge at 45 000 x g for 30 minutes at 0 degrees Celsius. The supernate obtained at the higher speed was mixed with sodium molybdate (50 mmol/L, final concentration). See table 1 for layout of experiment.

Receptor Determinations.

Estrogen receptor preparations were supplemented with 10 nmol of [^{3}H]estradiol per liter to determine total binding activity. Dextran-coated charcoal (5 g of charcoal and 0.5 g of Dextran C in 100 mL of 10 mmol/L Tris buffer) was added to remove unbound ligand, one volume of the charcoal slurry to nine volumes of cytosol. The radioactivity not absorbed was regarded as "bound" radioactivity. For liquid scintillation counting, "Scinti Verse E" fluor (Fisher Scientific Co., Fairlawn, NJ 07410), was used (2 ml in a 5 ml counting vial) and quench was automatically corrected for each sample on a Beckman Scintillation Counter with each sample being counted for 1 minute.

Size-exclusion Chromatography

Supernates from high-speed centrifugation were filtered through membrane filters with low protein-binding properties (Millex GV), to remove components that impair column performance. For isocratic elution, a flow metered pump (Model 112; Beckman Instruments, Berkley, CA 94710) was used to deliver buffer to a Spherogel-TSK exclusion column (G-4000,21.5 x 600 mm; Beckman Instruments) fitted with a guard column (Spherogel-TSK precolumns SW, 21.5 x 100 mm).

Samples were injected with a syringe-loaded injector (Model 210; Beckman Instruments) fitted with a 250-ul sample loop, then steroid receptors were eluted with P100 buffer containing 75 mL of dimethylformamide per liter. One mL fractions of column effluent were collected with a rapid response programmable fraction collector (Foxy;Isco Co.). The chromatographic system was maintained and operated at 2 to 5 degrees C. All samples contained 20 mM sodium molybdate and were run with and without protease inhibitors (see materials used).

Anion-exchange Chromatography

Supernates from high-speed centrifugation were filtered through membrane filters with low protein binding properties (Millex GV) to remove components that impair column performance. To deliver a gradient buffer system, a Waters 650 Advanced Protein Purification System fitted with a 250-ul sample loop was used. A Waters 650 System Controller delivered buffer and sample to a diethyl-aminoethyl (DEAE) column (21.5 x 100 mm, Millipore Corp., Waters Div., Milford, Mass., 01757). One ml fractions were collected on an Isco (Model 211) fraction collector.

Table 1. Experimental protocol for preparation of cytosols. All manipulations were performed at 0-4 degrees Celsius to prevent activation of receptor.

1. Sacrifice,dissect uteri

2. Homogenize (5 uteri/ml)

3. Slow spin (3200 x g, 15 min)

4. Collect supernatant

5. Fast spin (45000 x g, 30 min)

6. Divide into tubes B and C

7. Incubate with [^{3}H]-estradiol (according to following schedule)

	0-4deg	25deg	37deg
B1	120min		
B2		60min	60min
B3		120min	120min

	0-4deg	25deg	37deg
*C1	120min		
*C2		60min	60min
*C3		120min	120min

* add 10 ul/ml of protease inhibitors (see materials)

8. Remove unbound ligand (dextran-coated charcoal)

9. Divide samples in half

10. Size-exclusion and anion-exchange chromatography

11. Collect 1 ml fractions

12. Count activity

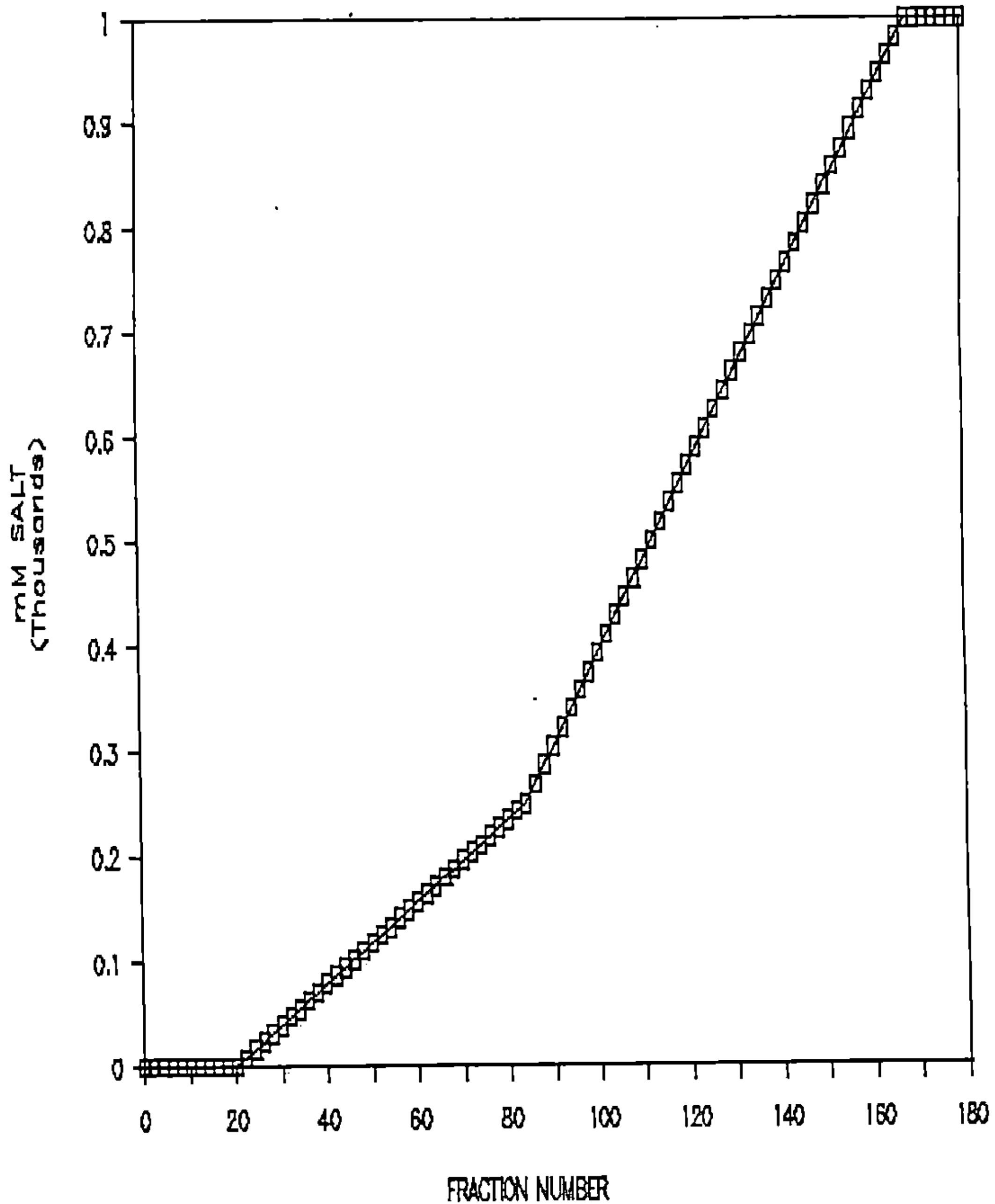

Figure 2. Salt gradient of Buffer B for anion-exchange chromatography. The gradient was determined using uracil as a marker and tracing its flow with an absorbance detector (280 nm).

Each gradient system consisted of a dual buffer. Buffer A and Buffer B contained identical biochemicals, except that Buffer B contained an additional salt, either potassium phosphate or sodium chloride. The ion-exchange system was equilibrated for approximately 20 minutes with Buffer A before the initiation of daily experimentation and between each run. Uracil was used alone as an absorbance marker (280 nm) to trace the gradient of salt in the system (Figure 2). See Table 2 for buffer systems used.

Table 2. Buffer systems used for anion-exchange chromatography. Bold print denotes salt used to interact with column and compete for binding sites with estrogen-receptor complex. EDTA, ethylenediamine-tetraacetic acid; DTT, dithiothrietol; DMF, dimethylformamide.

P1000 SYSTEM		NaCl SYSTEM	
Buffer A	Buffer B	Buffer A	Buffer B
P10	P1000	20 mM Tris	20 mM Tris
1.5 mM EDTA	1.5 mM EDTA	1 mM EDTA	1 mM EDTA
1 mM DTT	1 mM DTT	1 mM DTT	1 mM DTT
7.5 % DMF	7.5 % DMF	7.5 % DMF	7.5 % DMF
pH 7.0	pH 7.0		1 M NaCl

Data Management

Lotus 1-2-3 software was used to manipulate data and to create graphs for ease in representation of peaks. It was also used to determine the position of peaks using the @max functions and to calculate the relative percentages of each peak.

RESULTS and DISCUSSION

Size-Exclusion Chromatography

Chromatographs show activation of Peaks A and B with little change in Peak C (Figures 3,4). There was an obvious transfer of proportion of Peak B to Peak A when the sample was subjected to an increase in time or temperature of incubation. The use of protease inhibitors caused a minor shift in proportion of Peak B to Peak A at a lower incubation temperature (Figures 5,6).

In both systems used, Peaks A, B, and C were found to elute at or near a common fraction. This was also found in previous work done by Pavlik *et al*. The peak representing the largest isoform, Peak A, eluted at fraction 13. A slightly smaller isoform, Peak B, eluted at fraction 18, and Peak C eluted at fraction 24. By running ligand without receptor, it was determined that ligand not removed by dextran-coated charcoal eluted at positions 28-40 (results not shown).

Size-exclusion chromatography, in this work and in previous experiments, has been used as a control to determine the extent of activation of estrogen and

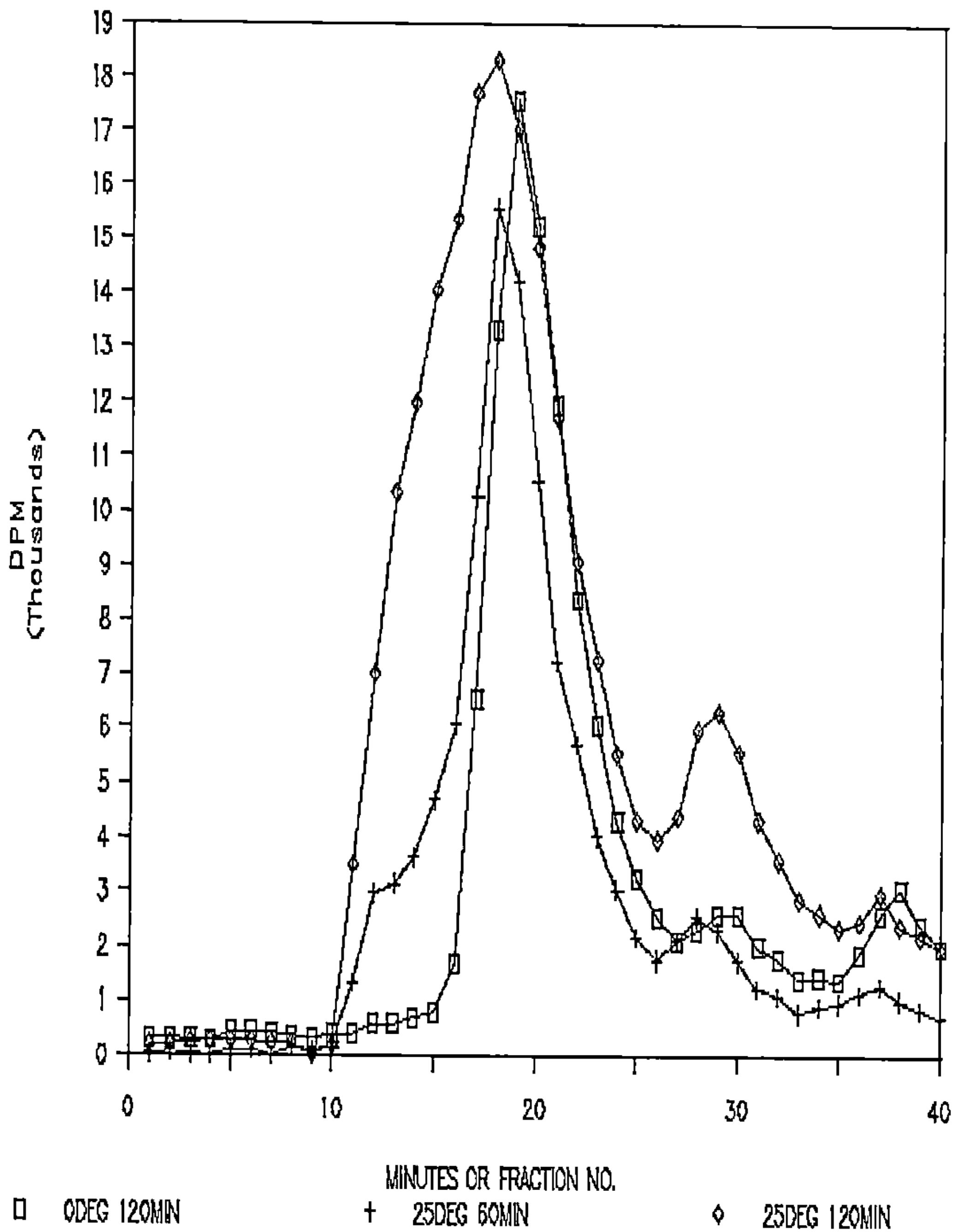

Figure 3. Size-exclusion chromatography showing activation of peaks due to increase in time and temperature of incubation. No protease inhibitors used.

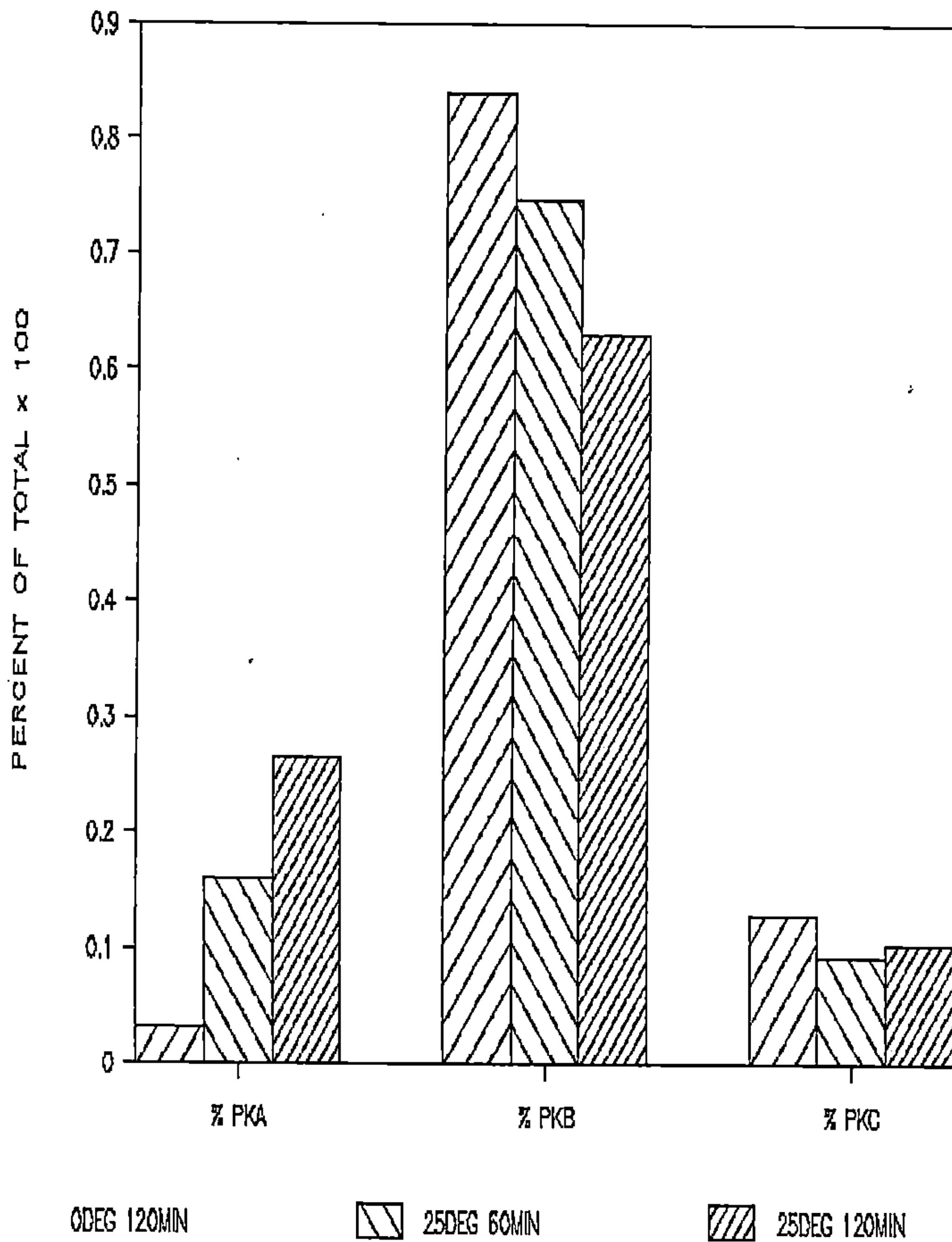

Figure 4. Relative proportions of peaks A,B, and C from figure 3. Note shift in proportion from Peak B to Peak A.

androgen receptors of various tissues [14-17]. Size-exclusion chromatography offers a rapid, reproducible means by which many tissues can be assayed in a relatively short time. Therefore, degradation and proteolysis are less of a contributing factor in the variability of chromatographs.

Anion-Exchange Chromatography

The P1000 system eluted 4 distinct peaks in each of the chromatograms. While all peaks eluted at less than 400 mM of salt, each peak could be found in particular areas of the salt gradient. A 0 mM peak was found at fraction number 6 in chromatograms of both systems. A ~47 mM peak occured very shortly after initiation of the salt gradient. An intermediate ~180 mM peak and a high salt species, or ~383 mM peak was eluted. Since the gradient is not constant, the fraction number corresponding to the mM of salt at which the peak eluted is used for statistical purposes (Table 3). The ~180 mM and ~383 mM species generally carried the majority of proportion (Figure 7,8). There was an apparent increase in peaks 0 mM and ~47 mM species with the use of protease inhibitors (Figure

9,10). The NaCl system also eluted 4 peaks in each chromatograph, however, in different positions than did the P1000 system. There was a 0 mM peak eluting at fraction number 6, as in the P1000 system. A ~55 mM peak eluted shortly after initiation of the gradient. Following a peak at ~234 mM salt was a high salt ~428 mM species. As in the P1000 system, the corresponding fraction numbers are used for statistical purposes (Table 3). Peaks 0 mM and ~428 mM carried the majority of proportion with a more even distribution of all isoforms with activation or the use of protease inhibitors (Figures 11-14).

Table 3. Simple statistical analysis of elution fractions. Abbreviations : x, mean; s.d., standard deviation.

P1000 SYSTEM (n=4)		NaCl SYSTEM (n=4)	
Peak (mM)	Fraction	Peak (mM)	Fraction
0	x=6, s.d.=0	0	x=6, s.d.=0
~47	x=32, s.d.=3.32	~55	x=34, s.d.=7.00
~180	x=66, s.d.=12.02	~234	x=80, s.d.=12.22
~383	x=100, s.d.=5.63	~429	x=104, s.d.=8.56

While there is some variability in the elution of fractions of peaks, ion-exchange high performance chromatography offers a rapid and fairly reproducible means by which proteins can be separated or purified. The early peak that eluted at fraction number 6 is

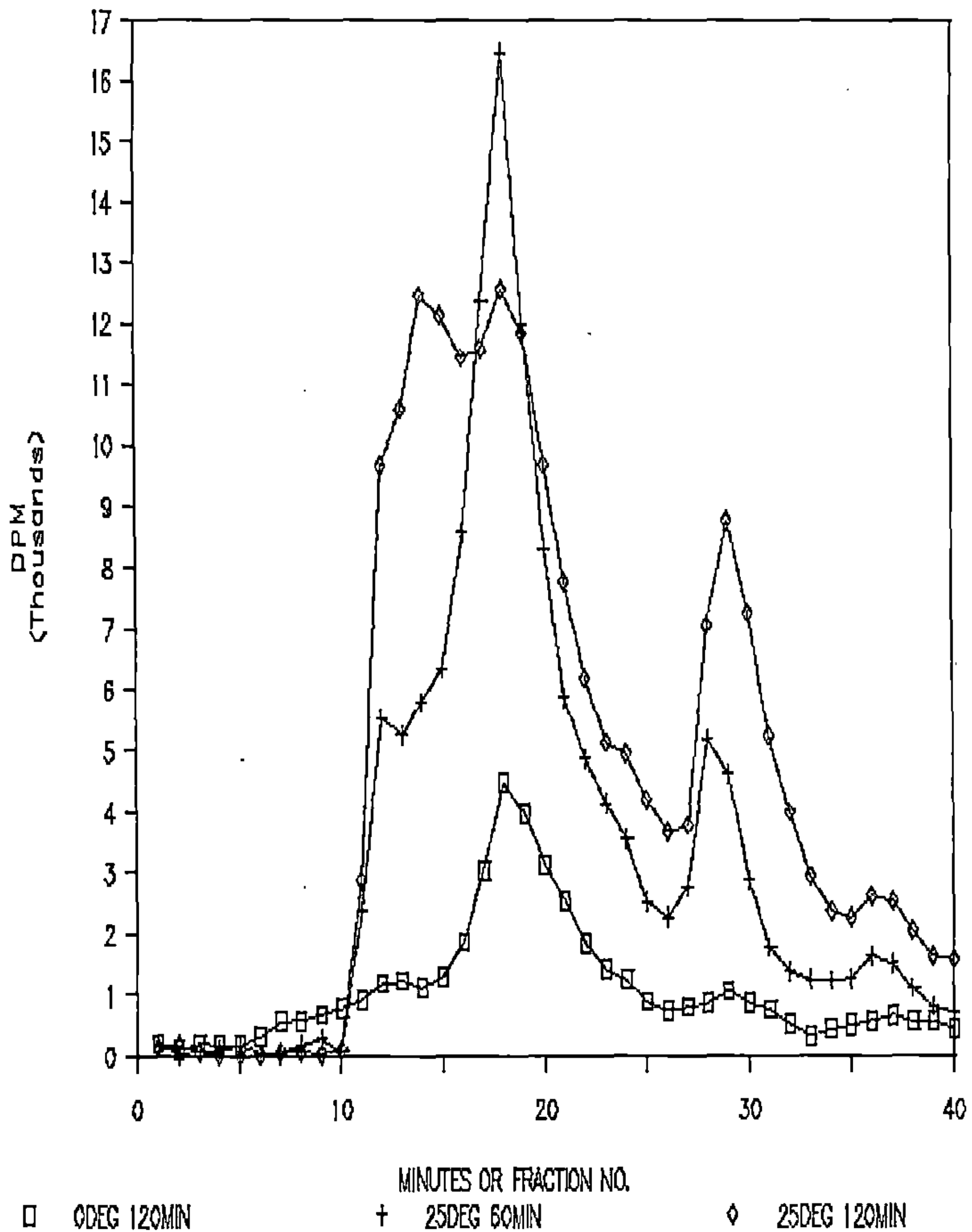

Figure 5. Size-exclusion chromatography showing activation of peaks due to increase in time and temperature of incubation. Protease inhibitors used.

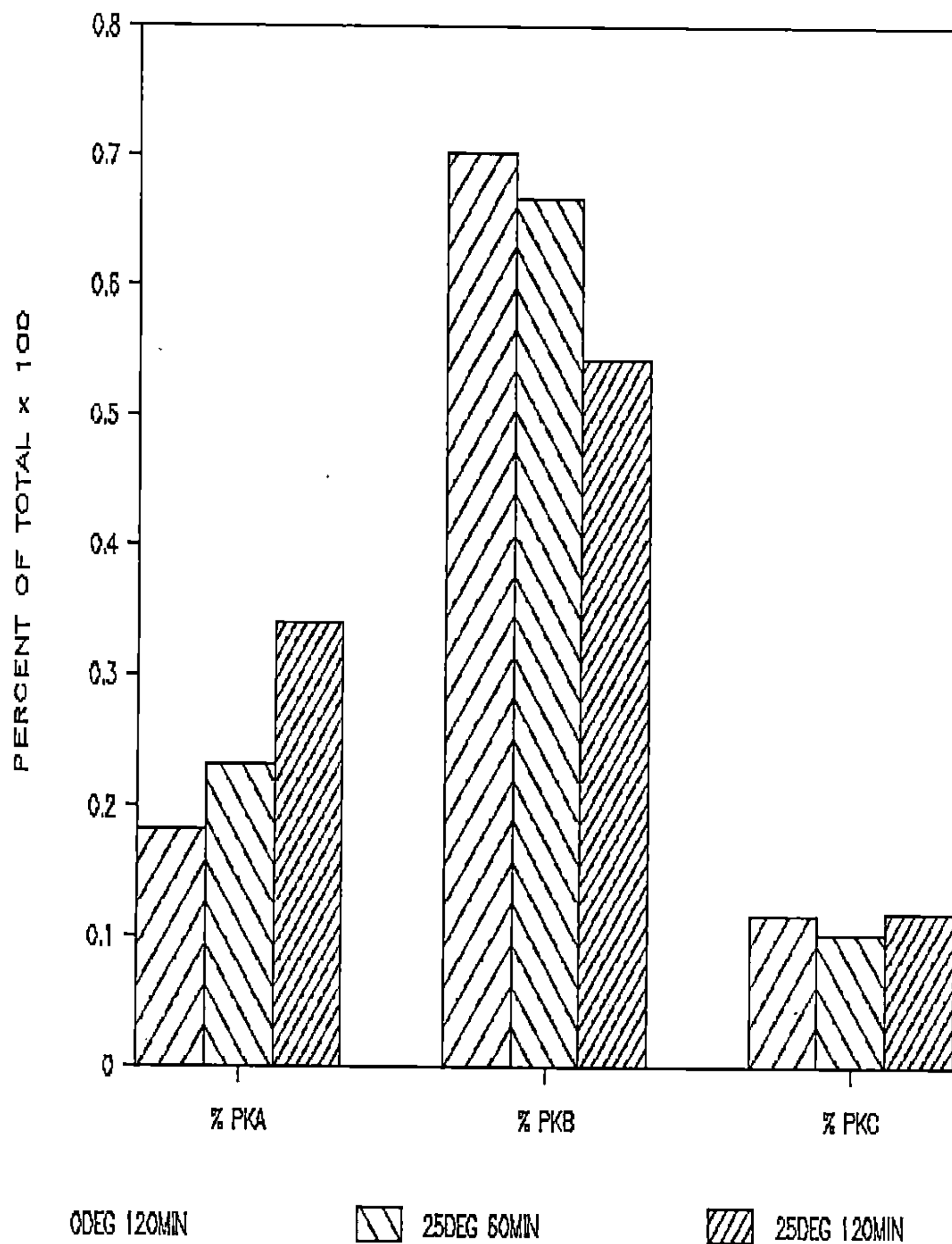

Figure 6. Relative proportion of peaks A,B, and C from figure 5. Note shift in proportion from Peak B to Peak A.

indicative of proteins which do not interact with the column on the basis of their surface charge, although on anion exchangers both the porosity and the hydrophobicity of the stationary phase may influence retention characteristics. This same early peak has also been found to elute with other anion exchange columns, such as the AX-300 [17]. Anion-exchange columns such as the AX-500 and the AX-1000 did not exhibit this early peak before initiation of the salt gradient. This early peak could be more precisely described by slowing down the flow rate of buffer through the system and by modifying the salt gradient.

While the murine estrogen receptor showed 4 isoforms, this is not the case in all animals and even varies with the use of different anion-exchange columns [17]. Wittliff et al have shown that a human breast cancer biopsy displayed 3 isoforms on the AX-300 and only one isoform on the AX-1000. A rat mammary gland showed 2 isoforms on the AX-300 and AX-1000, but only one on the AX-500. Each isoform eluted at a different position on individual columns.

It is evident from Table 3 that the NaCl system required more salt to elute receptor than did the P1000 system. This phenomenon can be described in light of the

fact that the P1000 system has a higher negative charge than does the NaCl system. Also worth mentioning is the large variability in the ~180 mM and the ~234 mM isoforms of the P1000 and the NaCl system, respectively. This third peak could be more susceptible to proteolysis than the other peaks. The elution characteristics of ligand without receptor (results not shown) demonstrated little or no interaction with the DEAE column. Ligand not removed by dextran-coated charcoal showed poor resolution and added little, if any, to isoform peaks.

While recovery of receptor was generally good (Figure 15), several chromatograms showed poor recovery. This low recovery can be attributed to at least 2 factors, namely (a) a retention of receptor molecule on the column or (b) a loss of ligand or "stripping" of the ligand from the receptor [17]. An answer to these problems may be provided through the use of affinity labeled ligands, post-column labeling, and/or monoclonal antibodies to the receptor molecule [17].

This work has demonstrated for the first time that the isoforms of the murine estrogen receptor can be described using ion-exchange high performance liquid chromatography. These experiments are the first in a long line of experiments designed to ultimately describe the ionic charge of the murine estrogen receptor. The

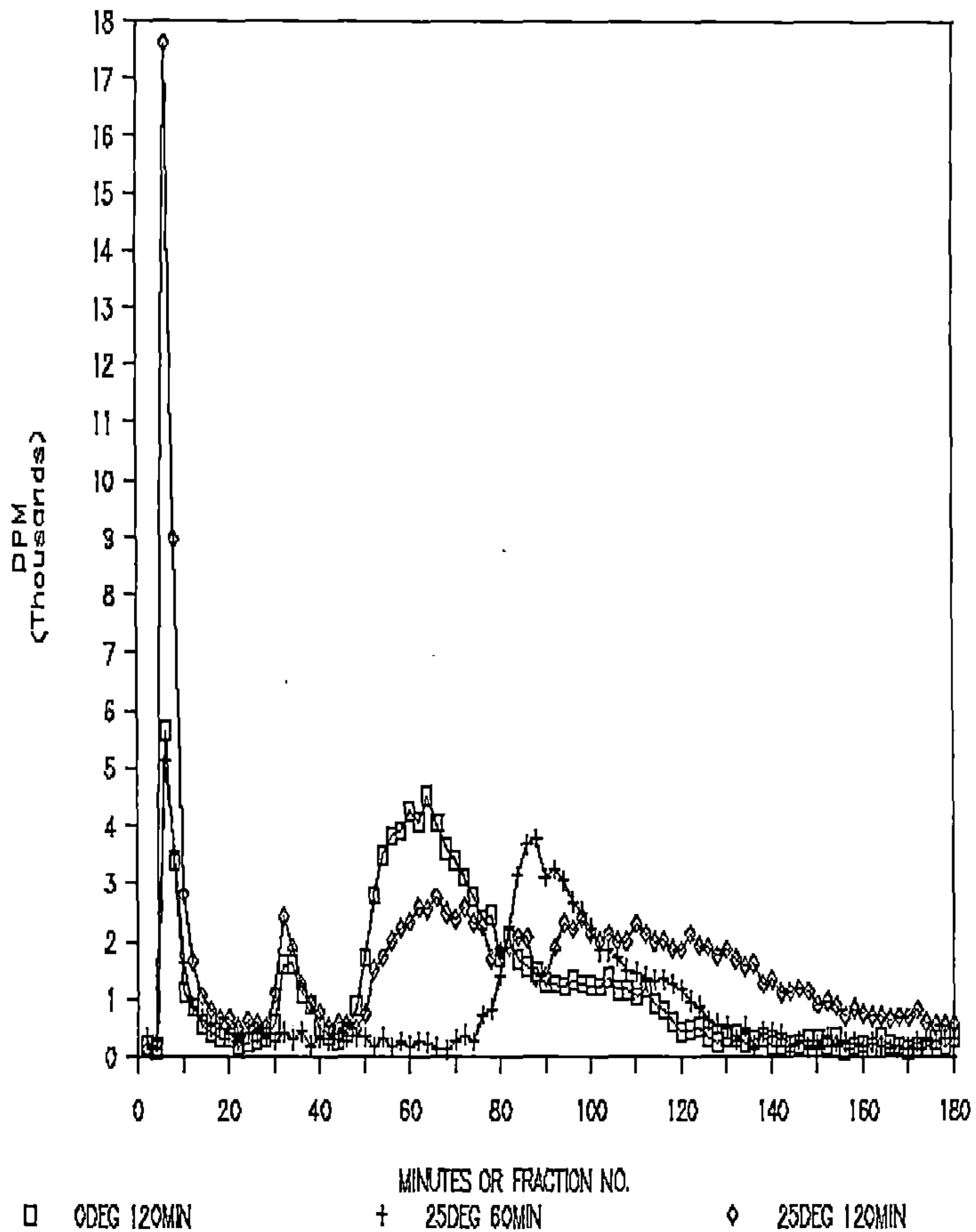

Figure 7. Anion-exchange chromatography showing activation of receptor due to increase in time and temperature of incubation with P1000 system. Note fraction 6, which eluted in every chromatogram.

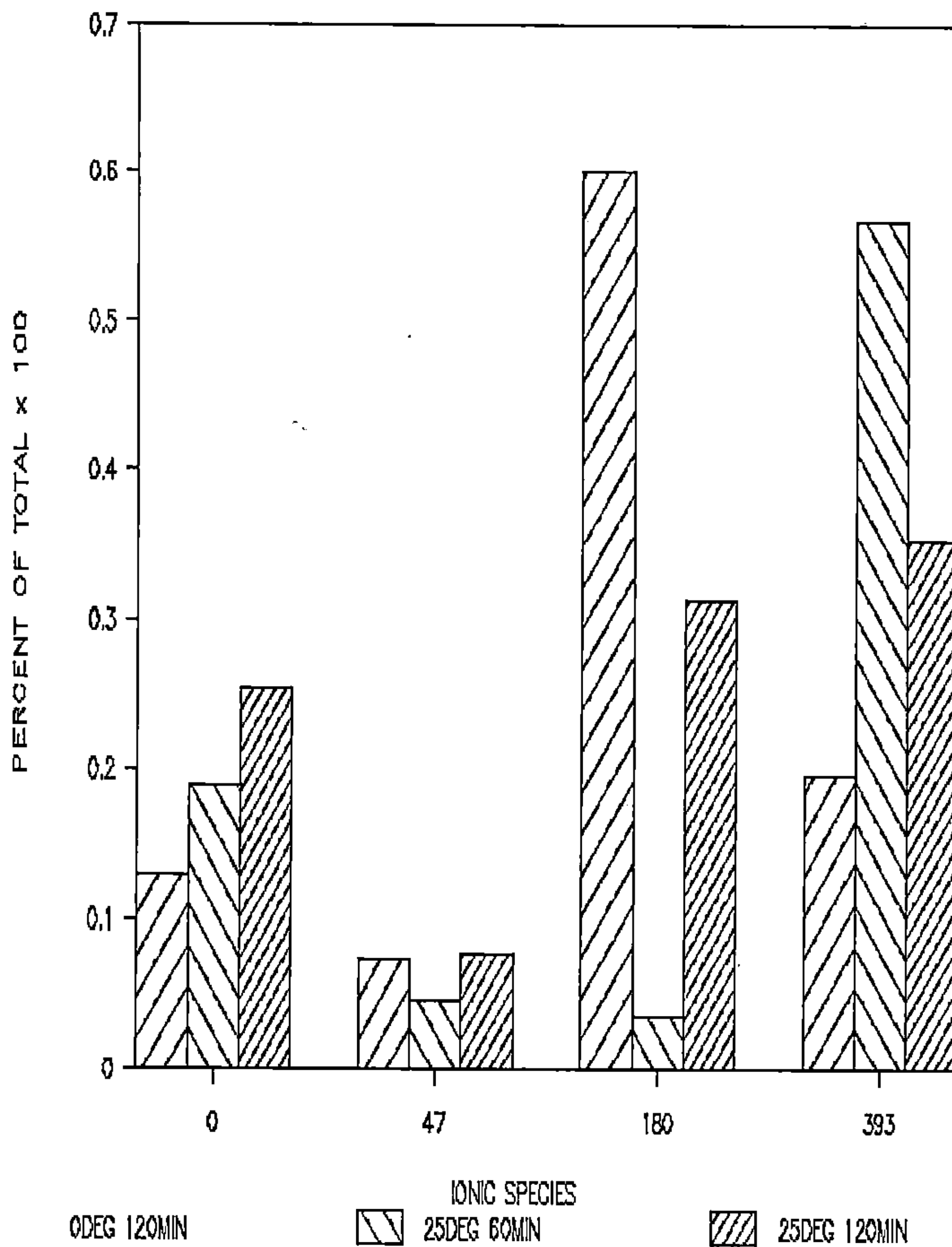

Figure 8. Relative proportions of isoforms from figure 7. No protease inhibitors used.

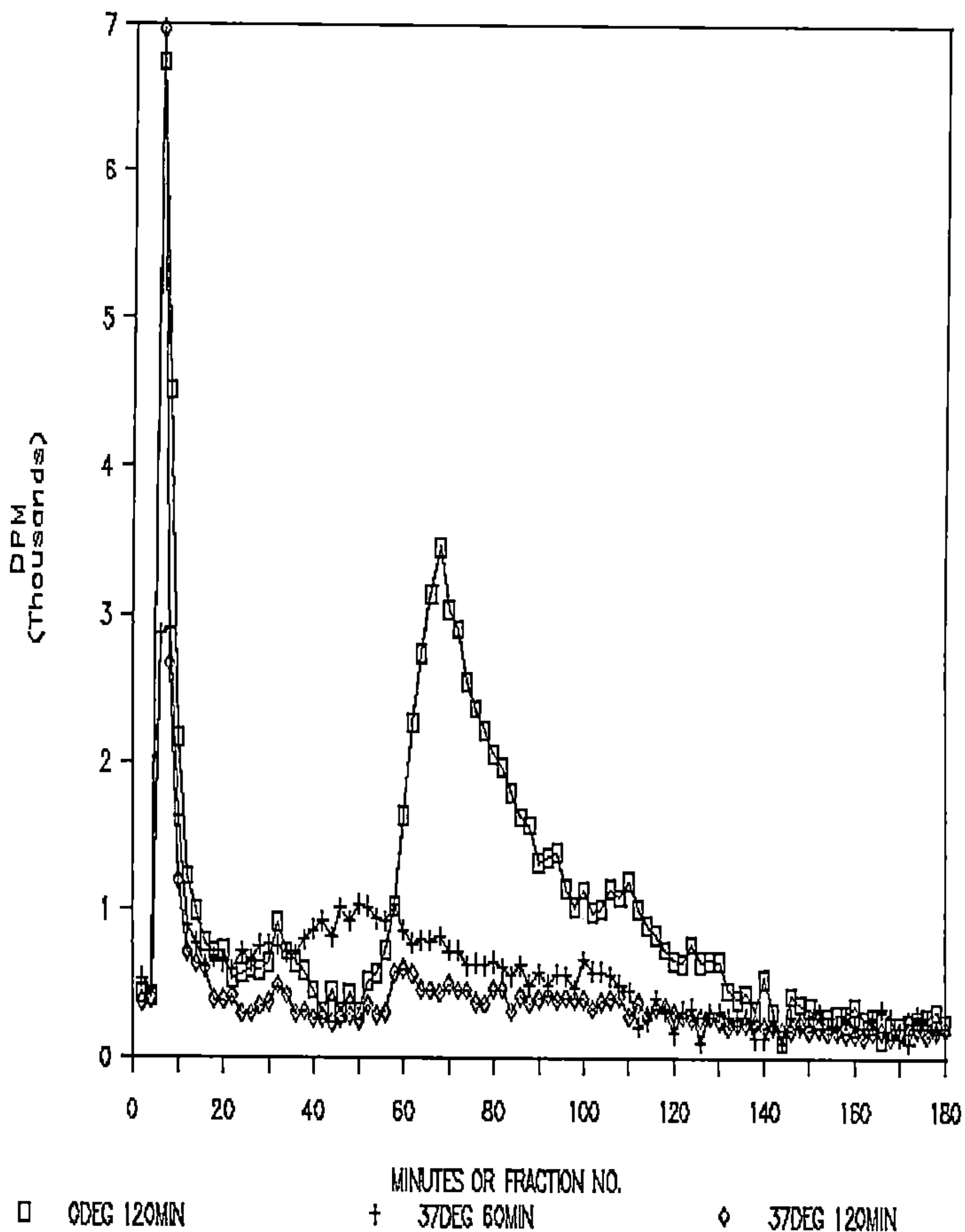

Figure 9. Anion-exchange chomatography showing activation of receptor due to increase in time and temperature of incubation (37 deg) with P1000 system. Protease inhibitors used.

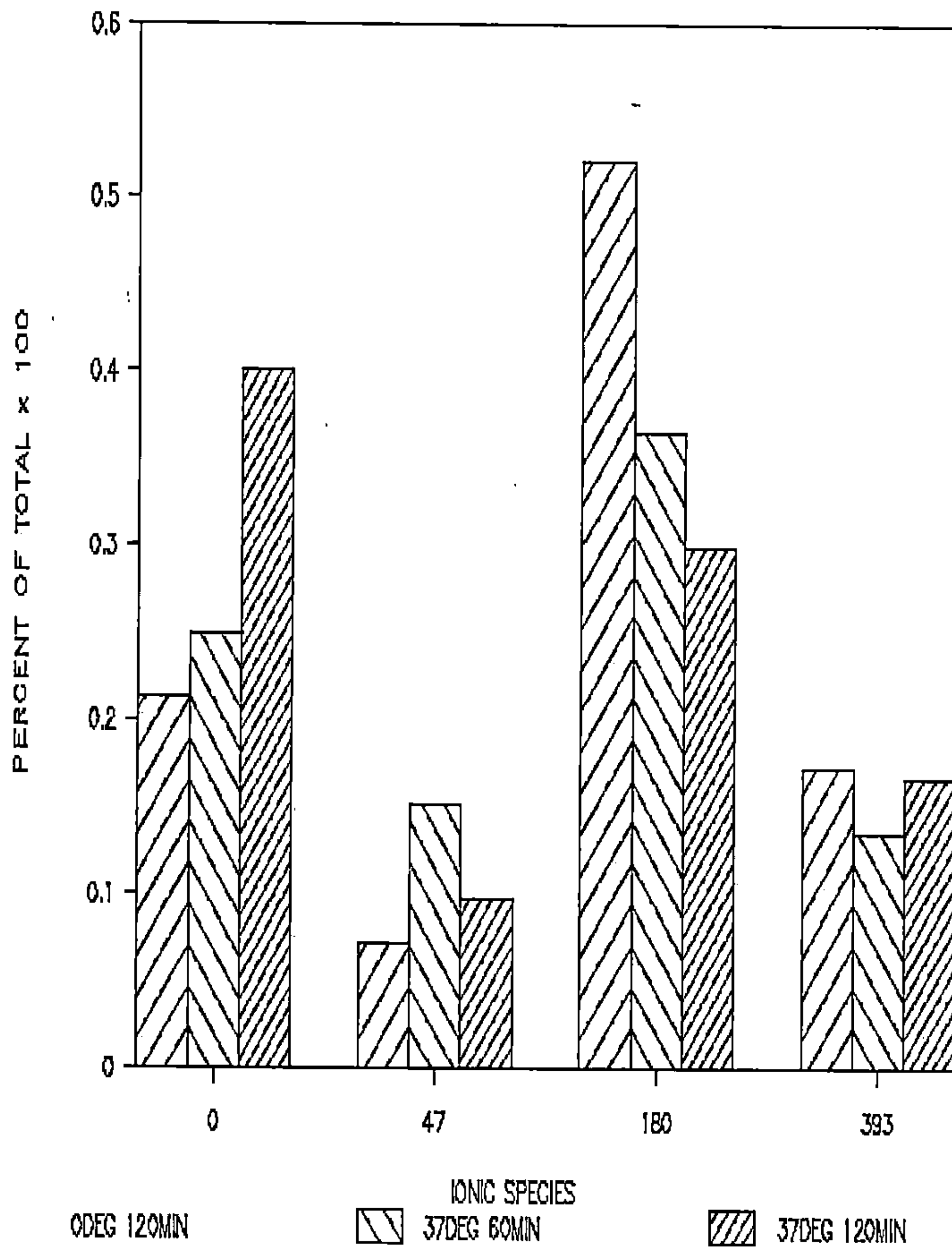

Figure 10. Relative proportions of isoforms from figure 9. P1000 system with protease inhibitors.

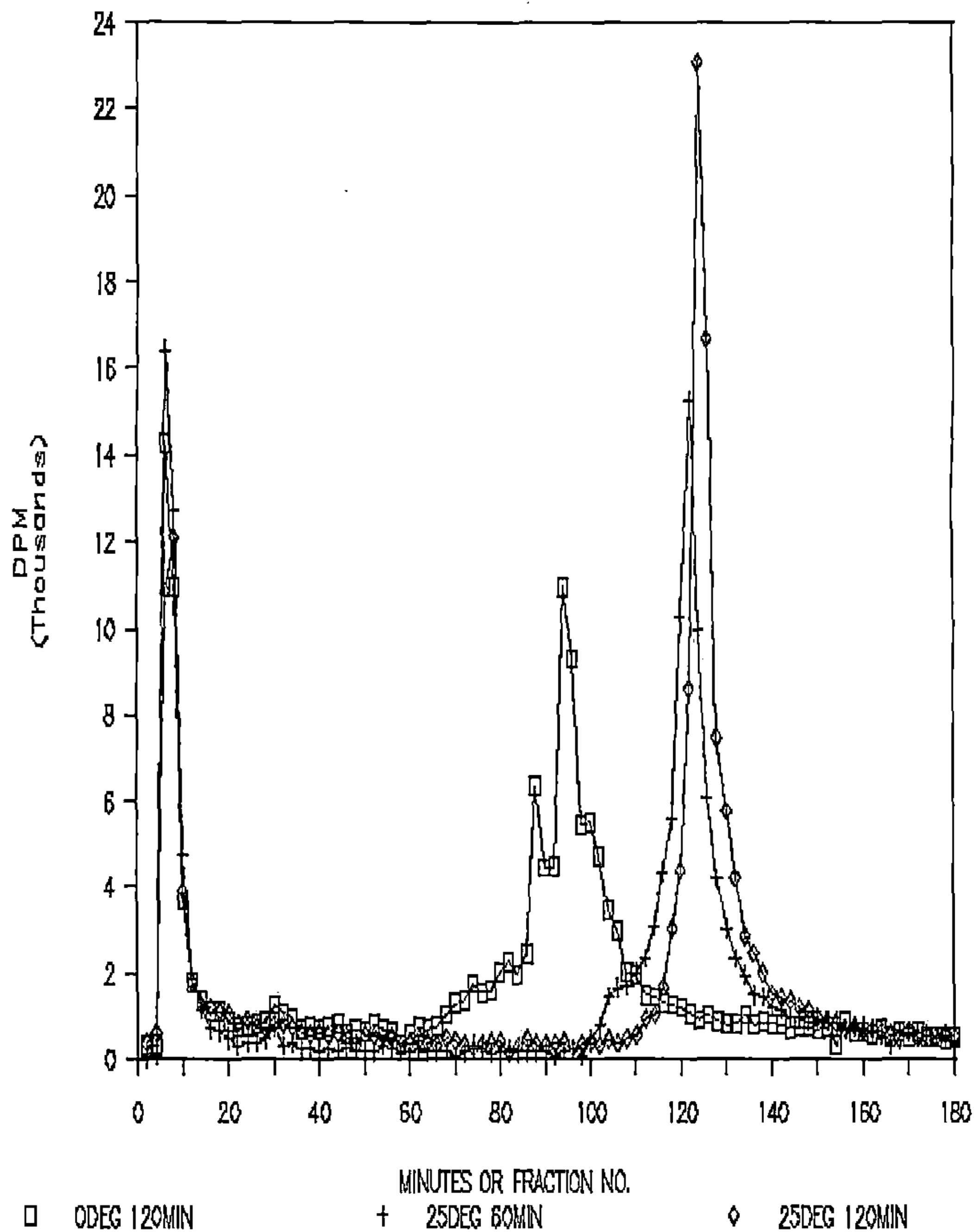

Figure 11. Anion-exchange chromchromatography showing activation of peaks due to increase in time and temperature of incubation. NaCl system without protease inhibitors.

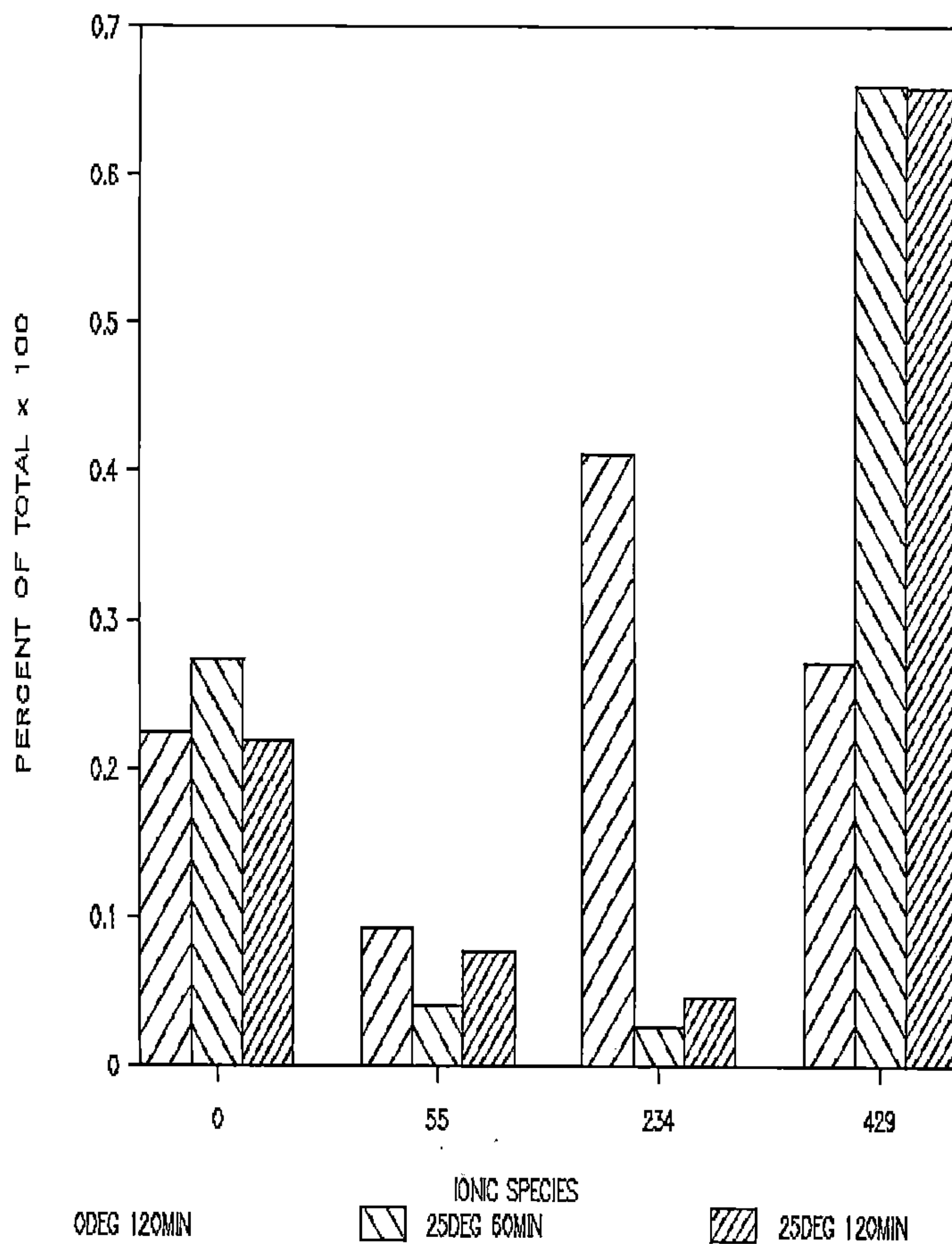

Figure 12. Relative proportions of isoforms from figure 11. NaCl system without protease inhibitors.

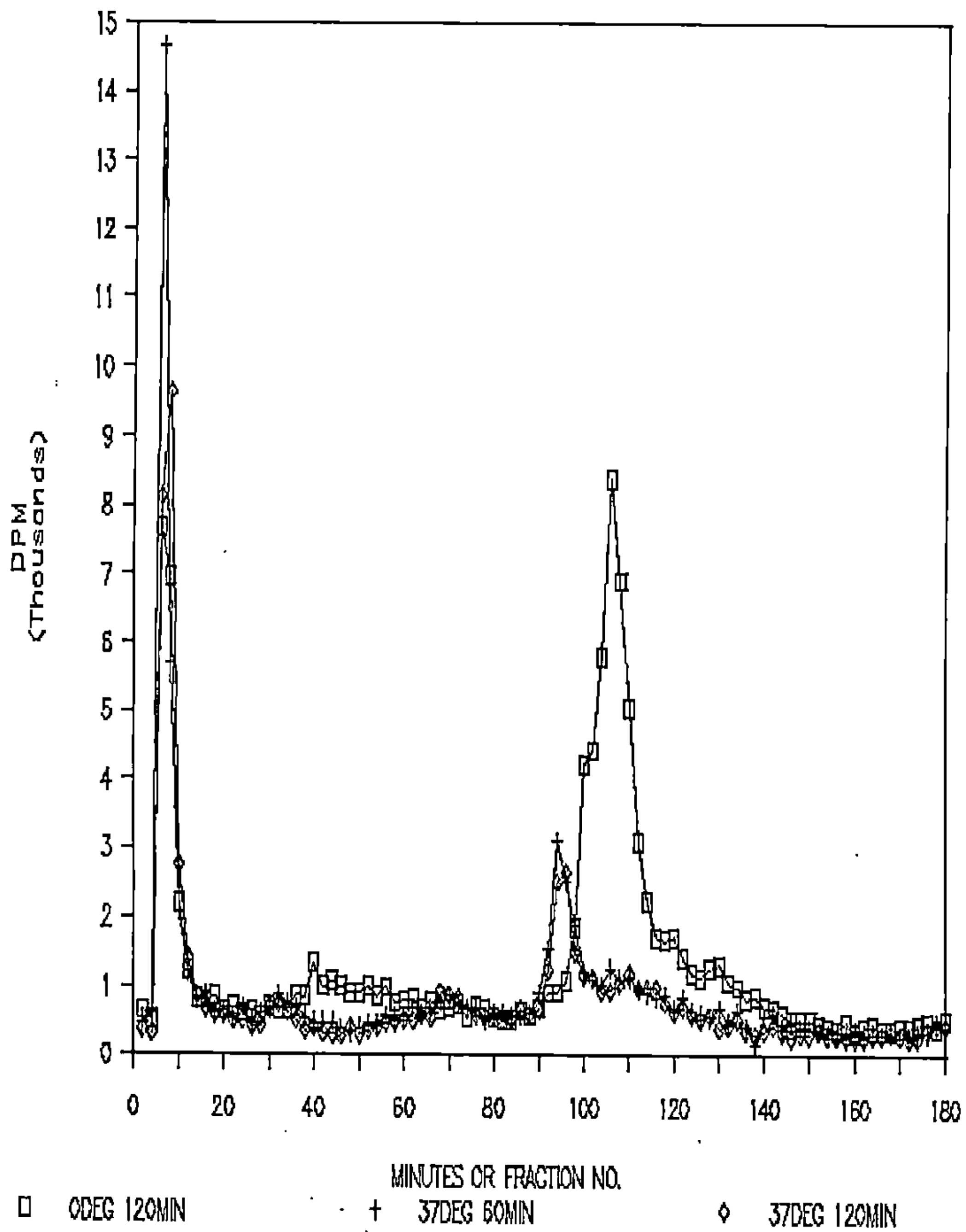

Figure 13. Anion-exchange chromatography showing activation due to increase in time and temperature of incubation. NaCl system using protease inhibitors.

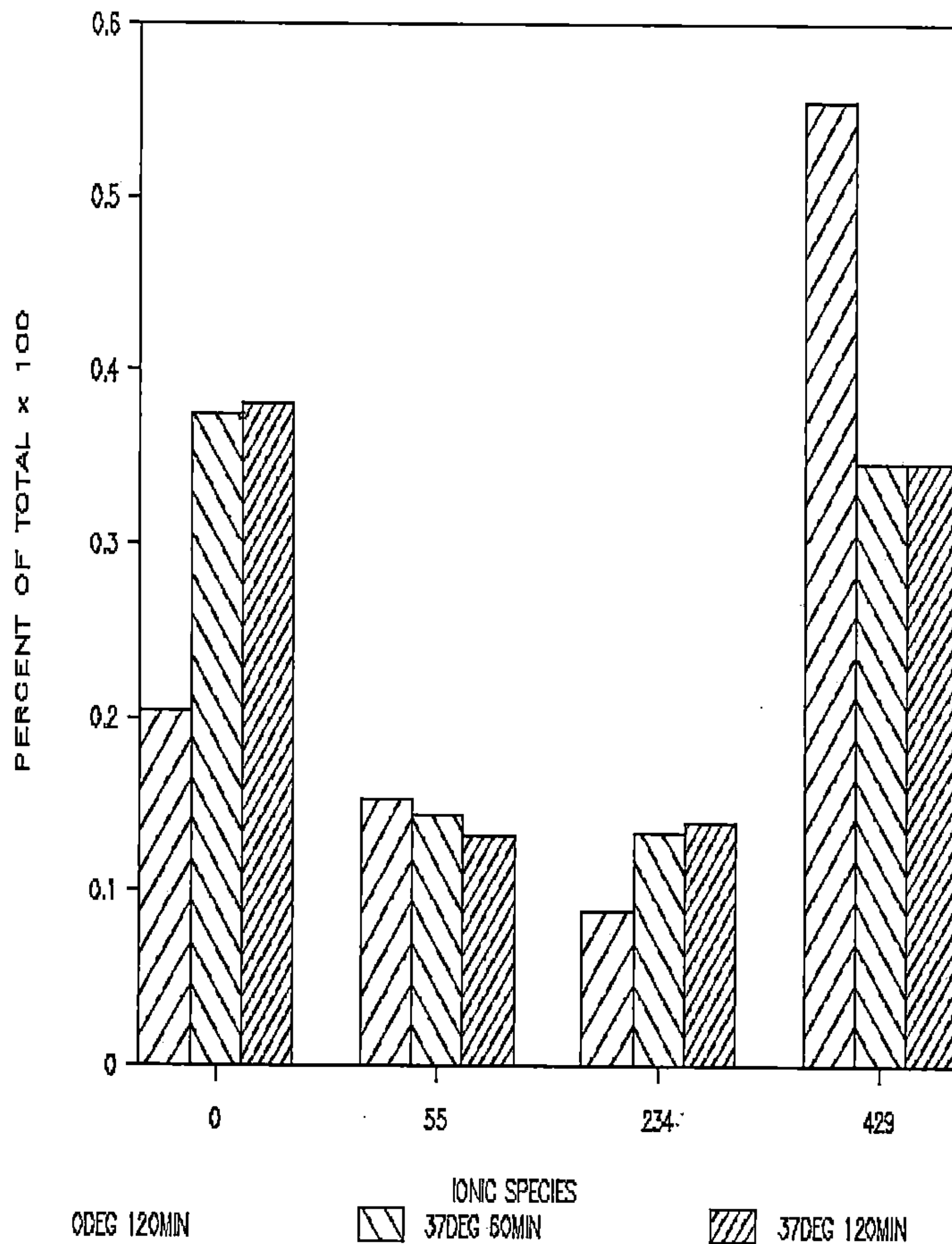

Figure 14. Relative proportions of isoforms from figure 13. NaCl system with protease inhibitors.

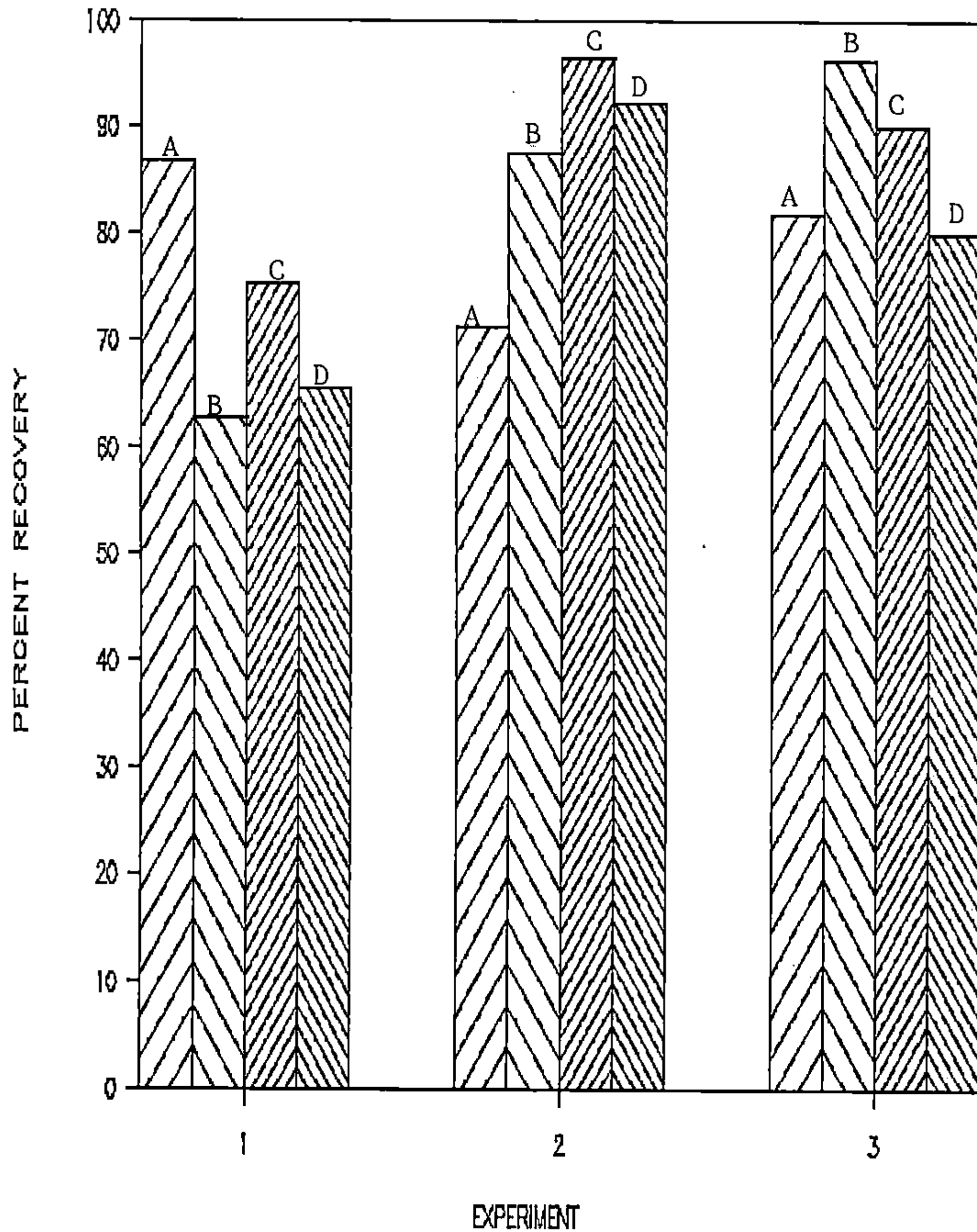

Figure 15. Recovery of receptor from anion-exchange column. Experiment 1-3: Refer to figures 9-14 for time and temperature of incubations. Bars A,B,C, and D coincide with figures 7,9,11, and 13, respectively.

work described here will serve as an aid not only in describing the ionic forms of the murine estrogen receptor, but will also help to determine the best buffer system in eluting the various ionic forms.

Much work has yet to be done to determine the validity of this work. For example, the effects of sodium and potassium on the murine estrogen receptor have yet to be described, for it is well known that many enzyme systems are dependent on sodium and potassium. With more experimentation, an ionic profile can be made of the estrogen receptor which will lead to a more precise assignment of endocrine therapies for patients suffering from estrogen dependent cancers.

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