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The affinity gel isolation of anthracycline receptive materials from the mouse heart

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THE AFFINITY GEL ISOLATION OF ANTHRACYCLINE RECEPTIVE MATERIALS FROM THE MOUSE HEART

by

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DISSERTATION

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ABSTRACT

The Affinity Gel Isolation of Anthracycline Receptive Materials from the Mouse Heart

The anthracycline antibiotics daunorubicin and adriamycin are both highly active anti-tumor agents in a variety of animal and human malignancies. However, in many instances the clinical effectiveness of these drugs has been limited by very serious cardiotoxicity. In humans, the myocardial effects of these agents include a reversible alteration in the electrocardiogram which may occur at any dose level and more importantly, cardiomyopathy leading ultimately to irreversible, sometimes fatal congestive heart failure.

Daunorubicin and adriamycin bind strongly to double stranded DNA and interfere with DNA and RNA metabolism both *in vivo* and *in vitro*. It is generally believed that the inhibition of DNA replication and DNA-dependent RNA synthesis are responsible for the cytotoxic effect and the anti-mitotic action of the drugs. However, it is not clear why the heart, an organ which does not show significant DNA replication, is the primary site of drug toxicity.

In this study, an attempt is made to determine if there exists, and if so, to isolate non-nucleic acid "receptor" in the mouse heart using affinity gels. A protein complex was eluted off the daunorubicin affinity gel after the gel had been incubated with nucleases-treated mouse heart homogenate. The isolated substance appears to be present mainly in the 100,000g supernatant fraction of the heart homogenate. From Sephadex and SDS-polyacrylamide gel electrophoretic

analyses, the key drug-recognizing protein has an apparent molecular weight of approximately 140,000 and appears to have a subunit structure. This material recognizes daunorubicin and adriamycin specifically and not daunomycinone, the first inactive metabolite of daunorubicin. It does not have an affinity for other amino compounds such as histamine, glucosamine and norepinephrine. Since the substance appears to be a cardiac receptive substance for the cardiotoxic anthracyclines, binding studies of anti-tumor anthracyclines with this receptive material in comparison with their in vivo cardiotoxicity may, in the future, provide a rational basis for synthesis and selection of non-cardiotoxic anthracycline analogs.

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INTRODUCTION

ANTHRACYCLINE ANTIBIOTICS

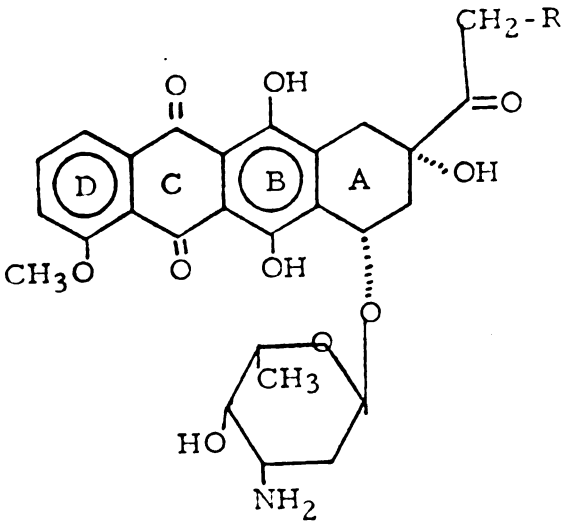
Unlike many other microorganisms which exist in a more or less steady-state ecologic niche, streptomycetes can be found in widely divergent environments. Their ability to deal with a vast variety of substrates and competing microorganisms become critical factors in their survival. Since the isolation by Waksman and Woodruff of actinomycin from Streptomyces antibioticus in 1940 (1) and Streptomycin from Streptomyces gresius in 1944 (2), the Streptomyces have received major attention from the microbiologists and the medical professions. Innumerable isolates from soil samples, taken from all parts of the world, have been systematically scrutinized for the production of antibiotics. They have yielded over five hundred different compounds from which more than fifty have found practical applications in human and veterinary medicine, agriculture and industry, including such important therapeutic agents as chloramphenicol, cycloheximide, novobiocin, nystatin and the tetracyclines.

In 1957, a red pigment-producing colony was isolated from a culture of soil from Puglia, a region of southern Italy. This microorganism was christened Streptomyces peucetius according to the name of one of the old tribes in that area. The antibiotic produced by this strain was codiscovered and named both rubidomycin and daunomycin; as a compromise, it is now designated as daunorubicin. As soon as the chemical structure of daunorubicin was clarified and

its antitumor activity demonstrated, a great deal of effort was expended in the isolation of other similar compounds from the Streptomyces species. Taking advantage of the high rate of either spontaneous or induced mutation in S. peucetius, a group of microbiologists headed by M. Ghione in Italy isolated Italian variant strains from this species. This eventually led to the discovery of a new compound: 14-hydroxy-daunorubicin (adriamycin) from a variant strain S. peucetius var. caesius. This marked the unveiling of the development of a new class of very potent anti-cancer chemotherapeutic agents: the antitumor anthracyline antibiotics.

In a series of papers published by an Italian group led by F. Arcamone in the 1960's, the structure of daunorubicin was deduced using various chemical identification techniques including absorption spectrophotometry, specific chemical degradation methods, nuclear magnetic resonance, mass spectrophotometry and optical rotation measurements (4-8). Primarily based on the correlation with that of daunorubicin, the structure of adriamycin was finally elucidated in 1969 (9). Figure 1 shows the basic structure of these compounds. They are composed of a tetracylic anthraquinone chromophore (Naphthacequinone) attached to a six-carbon sugar, daunosamine, through a glycosidic linkage. Daunorubicin and adriamycin differ only in one chemical group; the C-14 methyl of daunorubicin is hydroxylated in adriamycin.

FIGURE 1



R=H=Daunorubicin (Daunomycin)
R=OH=Doxorubicin (Adriamycin)

THE STRUCTURES OF DAUNORUBICIN AND ADRIAMYCIN

EFFECTIVE ANTI-CANCER AGENTS

Since the initial paper published in 1963 reporting significant antitumor activity of daunorubicin in ten experimental tumor systems which included both ascites and solid tumors (10), numerous studies have been done with both daunorubicin and adriamycin showing their potent inhibitory effects on the growth of a variety of animal and human malignancies. Clinical activity of daunorubicin has been primarily demonstrated in acute leukemia (23-25). On the other hand, adriamycin is effective against a wide spectrum of solid tumors in humans in addition to its activity against disseminated neoplasms (26). As a class, solid tumors are most resistant to chemotherapy and many of the solid tumors responsive to adriamycin are poorly or non-responsive to other drugs. Table 1 provides selective data from a review by Blum and Carter on the clinical use of adriamycin against human cancers (27). A response was defined in this study as a greater than 50% reduction in the tumor mass except in the case of acute leukemia. While none of these response rates approach 100%, it is important to note that the results have been obtained in many cases from patients in which other treatments have previously failed. In an article published in 1975, Gottlieb compiled a table listing the responses to adriamycin as a single agent in solid tumors based on all published and reported results through the end of 1973 (27a) (Table 2). A significant point regarding these data is that they are based upon single-drug treatments. Many of the most effective chemotherapeutic regimens for cancer now are based on combinations

TABLE 1

	Response Rates	
Breast Cancer	36%	44/121
Sarcoma	26%	46/176
Lung Cancer	19%	44/229
Malignant Lymphomas	41%	61/147
Acute Leukemia	24%	47/195

The Antineoplastic Activities of Adriamycin (8)

TABLE 2

Diagnosis	Number of patients	Number of responses	% Response
Breast Cancer	345	129	37 %
Bone Sarcomas			
Osteogenic Sarcoma	81	18	22 %
Ewing's Sarcoma	63	27	37 %
Chondrosarcoma	17	1	6 %
(All bone Sarcomas	161	46	29 %)
Soft Tissue Sarcomas			
Rhabdomyosarcomas	79	21	27 %
Fibro- and Neurofibrosarcoma	38	9	24 %
Leiomyosarcoma	37	12	32 %
Synovial Cell Sarcoma	22	7	32 %
Liposarcoma	19	6	32 %
Angiosarcoma	16	7	44 %
Undifferentiated Sarcoma	16	3	19 %
Unspecified and Misc	130	31	24 %
(All Soft Tissue Sarcomas	357	96	27 %)
Malignant Lymphoma			
Lymphocytic Lymphoma (LSA)	80	35	44 % (9 % CR)
Histiocytic Lymphoma (RCS)	74	46	62 % (11 % CR)
Hodgkin's Disease	116	36	31 % (3 % CR)
Not specified and Misc	72	40	36 %
(All Lymphomas	342	157	46 %)
Bronchogenic Carcinoma			
Squamous Cell Carcinoma	70	16	23 %
Adenocarcinoma	55	10	18 %
Oat Cell Carcinoma	43	13	30 %
Large Cell Undifferentiated Carcinoma	40	7	18 %
Unspecified	162	22	14 %
(All Bronchogenic Carcinoma	370	68	18 %)
Genitourinary Cancers			
Bladder Cancer	121	32	26 %
Renal Carcinoma	73	6	8 %
Testicular Cancer	78	33	42 %
Prostate Cancer	27	5	19 %
Gynecologic Cancers			
Ovarian Carcinoma	84	24	29 %
Cervix Cancer	31	7	23 %
Uterine Cancer	9	3	33 %
Head and Neck Cancer			
Squamous Cell Carcinoma	107	20	19 %
Thyroid Cancer	39	16	41 %
Gastroenteric Adenocarcinoma	213	23	11 %
Malignant Melanoma	40	1	3 %

THE ACTIVITY OF ADRIAMYCIN ON SOLID TUMORS AS A SINGLE AGENT (27a)

of drugs, not the use of single entities. Indeed, adriamycin has become one of the most important chemotherapeutic agents in the treatment of malignant diseases in man.

CARDIOTOXICITY

As in the case of all cancer chemotherapeutic agents now used clinically, daunorubicin and adriamycin are extremely toxic compounds. They cause the inhibition of mitotic activity and selective damage in tissues that are highly proliferative in post-fetal life. These tissues comprise the proliferative compartments of cell renewal systems in which a large number of new cells are produced continuously to replace daily losses of mature components. Consequently, toxicities such as myelosuppression, stomatitis, alopecia, nausea and vomiting are usually observed in cancer patients undergoing treatment using these drugs. However, there is one toxic effect that makes the anthracyclines unique among the other agents used in the treatment of cancer. The clinical effectiveness of daunorubicin and adriamycin is, most often, limited by serious cardiotoxicity.

In humans, the myocardial effects of the anthracyclines are of two types. The first type involves an acute and often transient alteration in the electrocardiogram of the patients which may occur at any dose level (28-32). In a study done by Cortes (32), EKG abnormalities developed in 25 of 100 patients during adriamycin therapy. Such changes included non-specific ST-T changes, prolongation of Q-T interval, atrial flutter and fibrillation with atrioventricular block, low QRS voltage in limb leads and poor R-wave progression in chest leads. The arrhythmias are only rarely of clinical consequence and are not clearly related to the cardiomyopathy (28). The second type of myocardial toxicity is of more clinical importance. The

anthracyclines cause an insidious cardiomyopathy affecting a variable but significant number of chronically treated cancer patients (28-32). These patients will develop irreversible congestive heart failure eventually if this toxicity is not managed properly. The clinical manifestations of the cardiomyopathy are related to the cumulative dose administered. When less than 500 mg/M^2 has been given, the incidence of cardiomyopathy is reportedly less than 1%. Eleven percent of patients given between 501 and 600 mg per square meter are affected. Among those given more than 600 mg/M^2, the incidence exceeds thirty percent (33-34). It is for this very reason that a maximum cumulative dose of adriamycin at 550 mg/M^2 was recommended (35).

Since the introduction of the drugs into clinical use, a great deal of effort has been put into the development of safe and reliable clinical tests to predict on an individual basis the progress of the cardiotoxic effects induced by the drug treatment. Some of the techniques developed included serum enzyme level measurements, echocardiography, ratio of pre-ejection period to left ventricular emptying time, electrocardiography and endocardial biopsies (36-43). Unfortunately, to date, none of these methods have been proven altogether adequate (43a). Cardiotoxicity of the anthracycline antibiotics has also been demonstrated in a number of animal systems including the rabbit (44), the rat (45-47), the mouse (48-48a), the hamster (49), and the monkey (50).

Detailed ultrastructural evaluation of the affected myocardium from patients and animals receiving either daunorubicin or adriamycin

indicates that nearly all the subcellular organelles of the cardiac mycocytes are altered (44-51). For example, affected myocardium of rabbits exhibits myofibrillar, sarcotubular and mitochondrial changes (44). Dilatation of the sarcoplasmic reticulum and the transverse tubular system appear to be one of the early changes and correspond to the vacuolation of myocytes evident at the light microscopic level. Myofibrillar degenerative changes include loss of parallel orientation, disruption of the Z-band registry, spreading or widening of Z-band materials, fragmentation into component sarcomeres, lysis and paucity of myosin and actin filaments, disorientation of remaining myofilaments and eventual fibrous replacement. Mitochondrial damages appear to be a consistent finding in degenerating myocytes. Early changes appear to be characterized by the swelling of cristae and outer mitochondrial membranes. Eventually, mitochondria undergo shrinkage and condensation and appear as small oval electron-dense inclusions fractions of their original sizes. In general, nuclear alterations are not striking in degenerating myocytes. Nuclear pyknosis and fragmentation are not observed until late in the degenerative process, after most of the other organelles have disappeared. The most obvious nuclear change noted is the appearance of multiple, large, loosely arranged nucleoli in some myocardial cells.

The general picture of cardiac cell damage is very similar in the animal models and in humans based on autopsy results of patients who died from anthracycline induced cardiac insufficiency. Recently, with the use of the transjugular endomyocardial biopsy technique, Dr. M. Friedman of U.C. San Francisco was able to find evidence of specific

adriamycin induced myocyte injuries in patients with as little as 180 mg/M^2 of the drug, and the damage became progressively more severe with higher doses (42). This data further substantiated the idea that the myocyte damages observed in autopsy samples were indeed due to specific adriamycin toxicity on the heart and not non-specific changes associated with heart failure or autolysis happening to tissues after death.

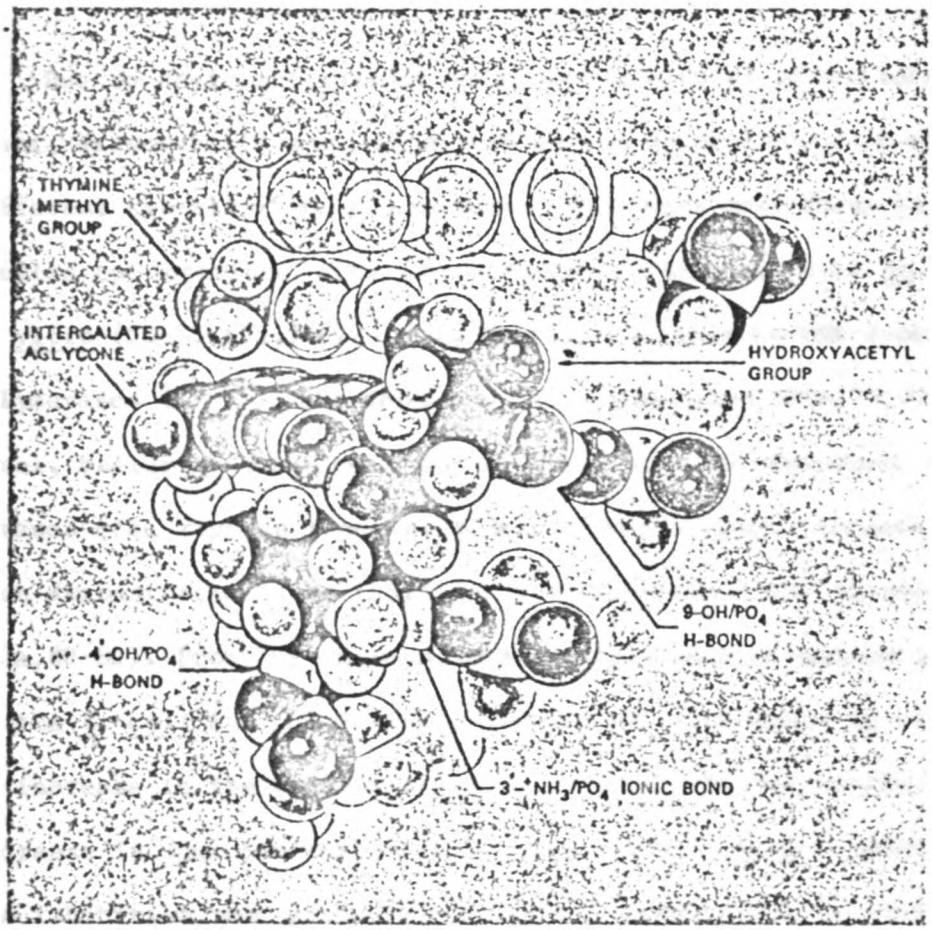
RECEPTOR SITE UNKNOWN - MANY POSSIBILITIES

Since the discovery of the anthracycline antibiotics as effective antitumor agents, substantial efforts have been put into the study of the interaction of these agents with various classes of biologically important molecules, in an attempt to obtain an understanding of their mechanism of action.

It is well established that daunorubicin and adriamycin form a tight complex with double stranded DNA *in vitro*. The drug-DNA interaction can be demonstrated by various biophysical changes occurring either in the drug molecule or in the DNA molecule. The binding of the drug to DNA results in a decrease in the absorption and a bathochromic shift in its ultraviolet-visible spectrum. The fluorescent intensity of the drug is also markedly reduced (54). Binding to DNA alters drug chemistry as well, inhibiting ionization of the phenolic hydroxyls at alkaline pHs and virtually eliminating the susceptibility of the drug towards polarographic reduction (54). This strong binding process saturates at about one drug molecule per five nucleotides for adriamycin and about six nucleotides for daunorubicin (55). On the other hand, the alterations of the biophysical properties of DNA upon antibiotic binding include: a decrease in the buoyant density of the DNA in CsCl gradients (56), an increase in the intrinsic viscosity of the DNA (57), a decrease in the sedimentation coefficient of the DNA (54-55), an increase in the melting temperature of the DNA (55) and the protection of the DNA from degradation by nucleolytic enzymes (58). Although many investigators have obtained evidence

suggestive of an intercalative mode of interaction between the anthracyclines and DNA (60-61), direct proof was not available until the study by Pigram in 1972, who examined fibres of DNA-daunorubicin complex by X-ray diffraction techniques (59). Based on their diffraction results and observations from molecular model building, together with the physicochemical data mentioned before, Pigram and his coworkers proposed a model for the daunorubicin-DNA complex in which the aglycone chromophore of the drug was largely overlapped by adjacent base pairs with the daunosamine side-chain projecting into the major groove of the helix where the amino group of the sugar interacted ionically with a phosphate moiety one base pair away from the intercalative site. The possibility of a hydrogen bond between the 9-hydroxyl and an adjacent phosphate was also suggested (59,62) (Figure 2).

In addition to their interaction with the nucleic acid component, daunorubicin and adriamycin have been shown to combine with a whole host of cellular components *in vitro*. For example, these agents were shown to have an affinity for phospholipids such as diphosphatidyl glycerol, phosphatidyl serine and phosphatidyl inositol (63) and increase the surface pressure of phospholipid monolayers spreading at the air-water interphase (64). Other cellular substances that have been shown to interact with either daunorubicin or adriamycin include: sulfated polysaccharides such as heparin and chondroitin sulfate (65), yeast t-RNA (66), actin and heavy meromyosin (67), tubulin (68) and even non-histone proteins isolated from rat cells (69). In fact, recently, daunorubicin attached to CH-Sepharose 4B affinity gel was



Proposed intercalative complex of adriamycin and helical B-form DNA as viewed from the major groove. Much DNA structure has been omitted to clarify specific bonding points. Four consecutive phosphate groups from one polydeoxyribosephosphate chain are shown extending from the upper right to the lower middle positions of the drawing. The phosphate ester links in this chain are in the 3' → 5' direction starting from the upper right. Part of a thymine–adenine base pair is shown immediately above the aglycone. The drawing is based on a CPK molecular model.

FIGURE 2 Pigram's Model of the Anthracycline-DNA Complex (8)

used successfully to fractionate non-histone proteins from rat leukemic cells (70).

Due to their interaction with such a variety of cellular components, daunorubicin and adriamycin have a multiplicity of effects on different aspects of cell functions. They have been shown in numerous studies to interfere with DNA and RNA metabolism both *in vivo* and *in vitro* (52-53,60a,71-72). *In vitro*, reactions of DNA-dependent DNA polymerases (73-77), DNA-dependent RNA polymerases (60a,75,77-79) from viral, bacterial and mammalian sources, and RNA-dependent DNA polymerases (the reverse transcriptases) from oncogenic viruses have all been demonstrated to be inhibited by daunorubicin and related analogs (60a,80-82). From the observation that daunorubicin-induced inhibition of the *E. coli* RNA polymerase catalyzed reaction could be overcome by an increase in the concentration of template DNA but unaffected by an increase in the concentration of the enzyme, Zunino concluded that the inhibition was probably due to an inactivation of the template function of the DNA and not to the direct inhibition of the enzyme (78). Mizuno and his coworkers, based on their data on the DNA polymerase and RNA polymerase isolated from mouse fibroblast tissue culture cells, arrived at similar conclusions (77). In contrast to its effects on nucleic acid synthesis, adriamycin was reported to have a negligible effect on protein synthesis in intact $A(T_1)Cl-3$ hamster fibrosarcoma cells although it did inhibit protein synthesis in a cell-free system consisting of polyribosomes, transfer RNA and enzymes (83).

The anthracyclines affect other aspects of cell function too. They have been shown to alter surface properties of cultured cells (84), perturb the magnesium ion-induced tubulin polymerization towards the dissociation of the polymer and inhibit microtubules assembly (68), inhibit heavy meromyosin and acto-heavy meromyosin Mg^{2+}-ATPase reactions (67), inhibit sodium-potassium-activated adenosine triphosphatase and cause marked alterations in sodium, potassium and calcium contents in cells (85-87). As might be expected from their effects on the morphology of the mitochondria in the myocytes of animals treated with adriamycin or daunorubicin mentioned previously, these agents interfere with mitochondrial functions. Because of the presence of tautomeric quinoid and hydroquinoid rings (ring B and ring C respectively) in the anthracycline antibiotics, their effect on the activity of coenzyme Q_{10}-enzymes in the mitochondria was investigated. Iwamoto's group reported in 1974 that both adriamycin and daunorubicin inhibited the activity of succinyl-oxidase and NADH-dehydrogenase isolated from beef heart mitochondria (88). Subsequently, Folker and Kishi observed that this inhibition could be prevented by the addition of coenzyme Q_{10} and other forms of coenzyme Q into the reaction mixture (89-90). In 1977, it was reported that the synthesis of coenzyme Q_{10} in mitochondria was also inhibited by the compounds (90a). Treatment of isolated mitochondria with anthracyclines in vitro resulted in impairment of mitochondrial functions measured as a lowering P/O ratio and a reduction in the respiration rate with succinate as the substrate (91), an inhibition of state 3 respiration oxygen uptake but not on state 4 respiration (92-93) and

the uncoupling of Ca^{2+} translocation from electron transfer reactions (93). Using mitochondria isolated from rat hearts, Bachmann concluded that adriamycin affected primarily oxygen consumption whereas daunorubicin had its main effect on oxidative phosphorylation (94). An impairment of mitochondrial function was also suggested when pulsating myocardial cells in culture treated with 1.7 µg/ml of adriamycin failed to respond to U.V.-microbeam irradiation of their mitochondria while 23% of the control untreated cells responded by an increase in the beating frequencies (95). However, since anthracyclines absorb strongly in the U.V. region, the mitochondria in the adriamycin treated cells might not have received the full U.V. dose of the microbeam irradiation. In contrast, the results obtained from attempts to demonstrate the effect of adriamycin and daunorubicin on mitochondria in vivo are not as consistent. For example, Casgill failed to see any alteration in the functions of mitochondria isolated from the hearts of rats treated with an LD_{100} dose of daunorubicin (91). In other studies, deterioration of mitochondrial functions was observed. Examining heart mitochondria isolated from rabbits subjected to a daily adriamycin dose of 0.8 mg/kg, Ferrero detected an impairment of respiration control due both to an increase of state 4b and a decrease in state 3 oxygen uptake which was reversible upon discontinuation of drug administration (96). These effects were dependent on dosing schedules and peak blood drug levels. Furthermore, these mitochondria consistently showed an increased permeability to added NADH while the ADP/O ratio, tissue contents of ATP, ADP and AMP measured in vitro were unchanged. Similarly, daily treatment of

rats with adriamycin was reported to cause a decrease in heart mitochondrial oxygen consumption after cumulative doses of 10 mg/kg with no effect on the ADP/O ratio (94). In the same study, daily treatment with 1 mg/kg of daunorubicin decreased both the mitochondrial oxygen consumption and the ADP/O ratio. On the other hand, a more recent study showed that the ADP/O ratio of heart mitochondria isolated from rat hearts treated daily with daunorubicin remained unchanged although the oxygen consumption was reduced (93).

Because of their extremely high affinity towards native DNA and their effect on nucleic acid metabolism both in vitro and in vivo, it is generally believed that the inhibition of DNA replication and DNA-dependent RNA synthesis are responsible for the cytotoxic effect and the anti-mitotic action of the antitumor anthracycline antibiotics. This notion is supported by the autoradiographic demonstration that daunorubicin enters cells and localizes mainly in the nuclei of both KB cells and rat liver cells (97). The concentrated appearance of drug specific fluorescence in nuclei (98-100) and in chromosome bands (101-103) of cells exposed to either adriamycin or daunorubicin further attested to their nuclei localization and their interaction with the genome of whole cells. A similar conclusion was arrived at using cell fractionation techniques (104-105). Very recently, it was shown that adriamycin inhibited the nuclear fluorescence of popidium iodide-stained human lymphocytic cells, providing yet another piece of evidence for the adriamycin-nuclear DNA interaction in mammalian cells (106). Finally, the theory that the biological effects of

these antibiotics are strictly related to the formation of a stable complex with DNA is strengthened by the observation that in a series of daunorubicin analogs, there is a good correlation between the inhibitory effect on nucleic acid synthesis in vitro, inhibition of proliferative activity and DNA binding ability as measured by physicochemical methods (52).

However, there is increasing evidence, especially from recent experiments, suggesting that the story might not be as simple as it was previously supposed. Earlier studies on the effects of daunorubicin and adriamycin on cultured cells indicated that the inhibition of mitosis induced by the drugs could occur to cells that had already completed DNA synthesis (107-109) and at drug concentrations which did not significantly affect DNA synthesis or the G_1 to S phase transition of the cell cycle (107,110). Silverstrini, using synchronized cultures of rat fibroblast, attempted to see if the anti-mitotic effect of the anthracyclines on G_2 cells was due to the inhibition of RNA synthesis in the G_2 phase when some of the necessary proteins for mitosis were synthesized (111). He found that the G_2 cells could be prevented from entering mitosis with doses of daunorubicin not affecting the RNA synthesis. He also noticed that daunorubicin could be added to the cell cultures after the RNA synthesis period in the G_2 phase and still maintain its anti-mitotic effect. A study on synchronized L-929 mouse fibroblasts showed that after daunorubicin treatment of the cells, the remaining mitotic figures exhibited an accumulation of mitosis in the telephase and disappearance of all other figures, suggesting that daunorubicin blocked some process

necessary for cells to enter cytokinesis (112). More recently, a new synthetic N-trifluoroacetyl substituted analog of adriamycin has been reported to be equally active in L1210 leukemia, P388 lymphocytic leukemia and solid sarcoma 180 in mice (113); and in other studies more active in a variety of murine tumor systems including both ascitic and disseminated L1210 leukemia, P388 leukemia, ascitic LSTRA lymphoma, advanced Lewis lung carcinoma and solid Ridgeway osteogenic sarcoma than the parent compound adriamycin (114-116) in spite of the observations that it was not metabolized to adriamycin in vivo (117-118); it was localized in the cytoplasm of cultured cells and not in the nuclei as in the case of adriamycin (100); it had a much lower affinity towards native DNA (119) and a 500 fold decrease in its ability to inhibit DNA polymerase in vitro (120). In addition, cross resistance of tumor cells towards daunorubicin, adriamycin and Vinca alkaloids, a class of potent anti-mitotic agents that is believed to act by a mechanism not involving the nucleic acid components of the cells, has been observed (121-125). Nor is it clear at all why the heart, an organ which does not show significant DNA replication, is the primary site of drug toxicity of a drug supposed to act by stopping DNA replication.

THE MECHANISM OF ACTION OF THE CARDIOTOXIC EFFECT OF THE ANTHRACYCLINE ANTIBIOTICS

What is causing the cardiomyopathy induced by the antitumor anthracycline antibiotics? One has to consider the possibility that this cardiac toxicity might not be a direct effect of the drugs on the heart cells. Indeed, the delayed cardiotoxicity induced in chronically treated rabbits has been related to an immune process as Fiorette reported that autoantibodies as well as sensitized spleen cells reacting specifically *in vitro* with rabbit heart cells could be detected and isolated from rabbits presenting myocardial alterations induced by adriamycin treatment (126-126a). However, it is possible that this phenomenon may only be the result of exposure of myocardial antigens secondary to some initial cellular damages. Furthermore, the importance of this phenomenon in cancer patients whose immune system is severely compromised is doubtful. At the annual meeting of the American Association for Cancer Research held in May, 1979, preliminary evidence was presented suggesting that adriamycin induced elevation in histamine and catecholamines in rabbits might be responsible for its cardiomyopathic effect (127). In this study, a combination of pretreatment and post-treatment of the animals with histaminergic blockers diphenhydramine (an H1 blocker) and cimetidine (an H2 blocker), and adrenergic blockers phentolamine (an alpha receptor antagonist) and propanolol (beta antagonist) was shown to successfully prevent the heart lesions observed in the control group. However, if released histamine and catecholamines are indeed the mediators for the

anthracycline heart toxicity, it would be difficult to explain why similar heart lesions are not observed in patients with chronic allergies such as "hay-fever" where the histamine and catecholamine levels are constantly elevated. In addition, the striking difference in the sensitivity of the cardiovascular system of different animal species towards histamine is well known to pharmacologists. The guinea pig is extremely sensitive to the action of histamine; the dog, the cat and the rabbit are moderately sensitive and the rat and the mouse are most resistant. For example, a cat will die of cardiovascular collapse after a dose of 3 mg/kg of histamine while a mouse can tolerate as much as 750 mg of histamine per kg body weight without serious toxicity (128-129). This casts further doubt on the histamine as mediator hypothesis as the anthracyclines induce the characteristic cardiomyopathy readily in different animal species including the rabbit, the mouse, the rat, the monkey and humans.

Perhaps the strongest evidence that points to the direct action of the anthracyclines on heart muscles comes from the experiments with cultured heart cells <u>in vitro</u>. Necco demonstrated that daunorubicin and adriamycin treatment of spontaneous beating heart cells of the mouse in cultures resulted in an arrhythmic beating pattern and an approximately 50% reduction in the beating frequency of the cells (130-131). Ultrastructural study of these cells showed alterations similar to heart cells isolated from animals chronically treated with adriamycin (132). Similarly, Petrovic and Seraydarian independently showed that adriamycin could inhibit the continuous rhythmic contractions of rat cardiac cells grown <u>in vitro</u> (95,133).

When he examined the intracellular ATP and phosphorylcreatine concentrations of these cultured cells, Seraydarian noticed an approximately 30% decrease as compared to control cells. The addition of creatine to adriamycin treated cells resulted in a several fold increase in the steady-state concentration of phosphorylcreatine. Therefore, it was concluded that the potential to maintain a high steady-state concentration of phosphorylcreatine was not impaired in these cells (133). Most recently, confluent cultures of heart cells isolated from newborn rats were studied with a polygraph recorder so that the beating rhythms of the cells could be examined (134). Significant increase in the incidence of arrhythmias was observed in cultures treated with adriamycin. In addition, the cells in the adriamycin treated cultures showed morphological changes not seen in cells of untreated controls or cultures treated with actinomycin D, 5-fluorouracil or thio-TEPA.

Knowing the high affinity of the anthracyclines towards DNA, one might suspect that it is the DNA binding property of these agents that is responsible for their injurious effect on the heart. Rosenoff demonstrated a decrease in the very low basal DNA synthesis <u>in vivo</u>, as measured by the ^{3}H-TdR incorporation into DNA isolated from mouse hearts, when the animals were treated with adriamycin (135). However, it was not clear whether this decrease of DNA synthesis was indeed a primary effect of adriamycin or was merely a reflection of cardiac injuries caused by other mechanisms. Fialkoff, in his study of the effect of adriamycin on neonatal rat cardiac cells in culture, observed a drop in the U.V.-stimulated DNA synthesis at high adriamycin

doses (10 μg/ml) although no effect was seen at low dose doses (1 μg/ml adriamycin) (136). However, the dose of the U.V. irradiation received by the DNA in the adriamycin treated cells and the control cells may not be comparable since adriamycin has been shown to localize in the nucleus (98-100, 104-105) and it absorbs strongly in the U.V. region. Similarly, a reduction in the DNA synthesis in mouse heart by adriamycin was demonstrated in an in vivo study by Formelli in 1978 (137). In this study, the ability of another clinically useful anti-neoplastic antibiotic, actinomycin D, to inhibit cardiac DNA synthesis was also investigated. Actinomycin D is an extremely potent anti-mitotic agent which has also been shown to intercalate native DNA and inhibit DNA and RNA synthesis, although in this case the RNA polymerase reaction is much more sensitive to the inhibition (138-141). No cardiotoxicity from actinomycin D administration has been observed in spite of its significant accumulation in the heart post-injection (142). The results of Formelli's experiment showed that actinomycin D, like adriamycin, also decreased DNA synthesis in the heart. In a recent study on rat heart cell cultures, morphological changes in cells related to the in vivo cardiotoxicity were seen only in adriamycin treated cultures and not in cultures treated with actinomycin D (134). Considering all these experiments, it seems doubtful that the inhibition of the nucleic acid synthesis by the anthracyclines is responsible for their cardiomyopathic action.

Since the heart is an extremely active organ, its dependency on energy production is remarkable. It is proposed that the anthracyclines induced cardiomyopathy might be the results of their interference

with the energy production system in heart muscle cells. Some successful attempts in reducing various aspects of the adriamycin induced cardiotoxicity in animal systems by coenzyme-Q administration have been reported in the past two years (143-145). However, Combs et al. failed to demonstrate an increase in the toxicity of adriamycin in animals under severe exercise stress, disputing the energy production defect hypothesis (146).

Finally, it has been suggested that the formation of superoxide radical in the presence of the anthracyclines which ultimately results in the conversion of membrane unsaturated fatty acids to lipid peroxides may be responsible for the cardiomyopathic action (147-150). Myers reported that tocopherol, an effective free radical scavenger, could reduce lipid peroxidation and the cardiac toxicity of adriamycin in mice, converting the LD_{85} for the drug to LD_{10} (152). More recent studies indicated that the protective effect of vitamin E (tocopherol) was most apparent during the first three weeks. However, the initial protection wore off with time and at sixty days, the death rates in both treated and untreated mice were the same (153). Very recently, N-acetyl-l-cysteine, another potent radical scavenger, was used successfully to reduce daunorubicin induced cardiomyopathy in mice (154). Despite all this suggestive evidence, a definite generation of reactive oxygen species in heart muscle cells has not been demonstrated.

THE HYPOTHESIS AND THE STRATEGY

From the evidence discussed previously, one is led to the hypothesis that the interaction of the antitumor anthracycline antibiotics with nucleic acid is probably not the important event in the generation of the observed cardiotoxicity, and that other "receptor(s)" is/are involved. Indeed, there are a variety of biologically important molecules that have been shown to interact with the anthracyclines *in vitro* (63-70) (see p. 13), although these interactions may be nonspecific in nature. The present study is designed to test the hypothesis that DNA is the only cell receptor of anthracyclines. A search was undertaken to try to detect and isolate materials other than nucleic acid from the heart that might bind strongly to the drugs and, very importantly, recognize them specifically. A very direct approach was employed, the drug molecule was used as a probe to search for materials in the heart that it recognized. The technique of affinity chromatography was best suited for this purpose.

EXPERIMENTAL

A) AFFINITY CHROMATOGRAPHY

Conventional biochemical methods of isolation and separation such as salt fractionation, adsorption chromatography, gel permeation chromatography, ion exchange chromatography and gel electrophoresis depend on gross physical and chemical differences between the substances to be separated. These methods are non-specific in nature and it is usually necessary to employ a series of these procedures in a multistage purification process. The procedures are frequently very laborious and time consuming, the separation accomplished is often incomplete, with extremely low yields. In contrast, the technique of affinity chromatography exploits the unique property of specific and reversible interactions between biologically important molecules. It provides opportunities for the isolation of substances according to their biological functions. The basic principle is to immobilize one of the components of the interacting system (e.g. the ligand) to an insoluble, porous support which can then be used to selectively adsorb, in a chromatographic procedure, that component (e.g. coenzyme, enzyme, etc.) of the bathing medium with which it can specifically interact. The greatest advantages of this technique lie in the simplicity of preparation and the rapidity in the separation; it is potentially a single step purification process; for example, a vitamin binding protein was purified over 10,000 fold in one step this way (158). The term affinity binding was originally used to describe the most obvious cases of such interacting systems: those

constituted of enzymes and competitive inhibitors. Today, after years of development and improvement, the technique has found applications in a wide variety of disciplines including immunology (antigen-antibody purification), biochemistry (purification of enzymes, nucleic acids, cofactors or vitamin binding proteins, repressor proteins, transport proteins and peptides formed by organic syntheses) and applied sciences such as pharmacology (drug and hormone receptor purification) (156-160).

B) THE SYNTHESIS OF THE AFFINITY GELS

I) The Choice of the Basic Gel Matrix:

The drug molecule must somehow be coupled to an insoluble solid support. Covalent coupling is much preferred to insure secure anchorage of the ligand. The way the ligand is attached to the support is a crucial consideration in affinity chromatography. Once the drug is attached covalently, it becomes derivatized and it is of utmost importance to anchor the drug molecule in such a way that its function would not be impaired. The biological activity of the ligand must remain unaffected after the coupling reaction for meaningful and relevant materials to be isolated. In addition, the coupling process must be carried out in conditions under which neither the drug nor the gel matrix is destroyed.

In this study, Affi-Gel-202 from the BioRad Laboratories (Catalog #153-3301 Capacity 20 µmole/ml) was chosen as the solid support material. Affi-Gel-202 has Biogel A15M as the basic gel matrix (molecular exclusion limit at 15 x 10^7 daltons) which is chemically crosslinked to provide stability to autoclaving and to chaotropic agents such as 3M thiocyanate, 6M guanidine HCl or 5M $MgCl_2$. A spacer arm of fifteen atoms ending in a carboxyl function was attached to the gel matrix (see Figure 3 for the structure of the gel). To minimize leakage problems, the spacer arm was attached to the agarose beads by a very stable ether linkage rather than the cyanogen bromide coupling commonly used to derivatize agarose supports for affinity chromatography. This particular support was selected for this study because of

Affi-Gel 202 from the Bio-Rad Company : Agarose- $OCH_2\overset{O}{\overset{\|}{C}}NH(CH_2)_3NH(CH_2)_3NH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}\text{-OH}$

1) $\bullet\text{---}\overset{O}{\overset{\|}{C}}\text{-OH} + NH_2NH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHNH_2 \xrightarrow{EDAC^*} \bullet\text{---}C{=}NNH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHNH_2$

2) $\bullet\text{---}C{=}NNH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHNH_2 + \text{Drug} \longrightarrow \bullet\text{---}C{=}NNH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHN{=}\overset{13}{C}\text{--(drug)}$

3) $\bullet\text{---}C{+}NNH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHNH_2 + CH_3\overset{O}{\overset{\|}{C}}CH_3 \longrightarrow \bullet\text{---}C{=}NNH\overset{O}{\overset{\|}{C}}(CH_2)_2\overset{O}{\overset{\|}{C}}NHN{=}C\begin{matrix}CH_3\\CH_3\end{matrix}$

* EDAC : 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride.

FIGURE 3 SYNTHESIS OF AFFINITY GELS

the following reasons. The gel matrix has a high degree of porosity which permits unimpaired entry and exit of large macromolecules throughout the entire matrix. This maximizes the concentration of ligand freely available to the macromolecules and thereby increases the possibility of isolating a large quantity of materials recognizing the ligand (161). Secondly, there is very little non-specific charge present in the basic gel components and this will reduce non-specific ionic interactions to a minimum. In addition, the diaminodipropylamine ($-NH(CH_2)_3NH(CH_2)_3NH-$) type spacer elements are generally less prone to hydrophobic interference than purely methylenic spacer segments (162). Thirdly, the gel beads are relatively stable mechanically and chemically under varying conditions of pH (pH 2-12), ionic strength and temperature (2-30° C). The gel is also resistant to the treatment of organic solvents including alcohol, dimethyl formamide, dioxane and pyridine. These features of the gel matrix were significant when daunomycinone affinity gel was synthesized as a control (see below) as well as providing opportunity to effectively use many potential eluting solvents and eluting conditions.

II) The Procedures for the Synthesis of the Affinity Gels

(A) Synthesis of the daunorubicin affinity gel:

(a) Succinyl dihydrazide (6.16 mmole) was dissolved in 4 ml of water and the insoluble fraction filtered off. The insoluble fraction was washed with an extra 1 ml of water. The filtrate and the washing were combined and adjusted to pH 4.5 with 3 N HCl. To the dihydrazide solution was now added 5 ml of Affi-Gel-202 (the gel was washed with approximately 50 ml of water before use). This is suspension A.

(b) EDAC (1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl) (5 mmole) was dissolved in 5 ml of water and the solution adjusted to pH 4.5 with 3N HCl. This is solution B.

(c) Solution B was added to suspension A and the mixture shaken at room temperature for 20 hr. The pH of the reaction mixture was maintained at pH 4.5-5.0 during the reaction.

(d) The product gel was collected by filtration and washed with water. This is product gel C.

(e) Product gel C was added to 0.1 mmole of daunorubicin HCl in 5 ml of water and the suspension shaken for 48 hr at room temperature. The product gel (red particle) was collected by filtration and washed with water extensively. This is product gel D.

By spectrophotometric absorption analysis, approximately 50% of the added daunorubicin reacted with the gel, giving a drug ligand capacity of 10 μmole per ml of affinity gel.

(f) The product gel D was added to 20 ml of 5% acetone solution and the suspension shaken for 20 hr at room temperature. The final product daunorubicin affinity gel was stored in 5 ml of daunorubicin solution (0.05 mmole) in the refrigerator until used.

(B) Synthesis of affinity gel - Daunomycinone ligand

(a) Product gel C was prepared as for daunorubicin affinity gel.

(b) A solution of 60 mg of daunomycinone in 10 ml of pyridine was mixed with product gel C in 3 ml of water and the mixture shaken for 9 days at room temperature.

(c) The product gel (red particle) was collected by filtration and washed with 200 ml of 50% pyridine in water and then 300 ml of water. This is product gel E.

By absorption analysis of the recovered drug washes, approximately 13 mg of daunomycinone reacted, yielding a drug-ligand capacity of 6 μ mole/ml affinity gel.

(d) The product gel E was added to 20 ml of 5% acetone solution and the mixture shaken overnight at room temperature. The gel was then washed extensively with water and stored in 5 ml of water at 4° C until used.

(C) Synthesis of affinity gel - Control gel with no drug attached:

(a) Product gel C was prepared as for daunorubicin affinity gel.

(b) The product gel C was added to 20 ml of 5% acetone solution

and the suspension shaken for 20 hr at room temperature. The product gel was then washed thoroughly with water and stored in 5 ml of water at 4° C until used.

(D) Synthesis of affinity gel - adriamycin ligand

(a) Product gel C was prepared as for daunorubicin affinity gel.

(b) Product gel C was added to 100 mg of adriamycin HCl in 5 ml of water and the suspension shaken for three days at room temperature followed by 16 days of shaking at 4° C (a total of 19 days of incubation). At the end of the incubation, the product gel (red particle) was collected by filtration and washed with water extensively. This is product gel F.

By absorption analysis, approximately 20 mg of adriamycin reacted with the Affi-gel-202, yielding a drug-ligand capacity of approximately 7 μmole per ml affinity gel.

(c) Product gel F was added to 10 ml of 5% acetone solution and the mixture shaken at room temperature for 20 hr. The product gel was then washed extensively with water and stored in 5 ml of 6 mg/ml adriamycin solution at 4° C until used.

In the synthesis of affinity gels, the carboxylic terminal of the spacer arm was first converted into an hydrazide function by reacting the affinity gel with succinyl dihydrazide (Aldrich Chemical Company 96% catalog #S550-2) in the presence of a water soluble carbodiimide, EDAC (BioRad Laboratories catalog #153-0990) (Figure 3, Step I). In an acidic environment, the carbodiimide (I in Figure 4) was

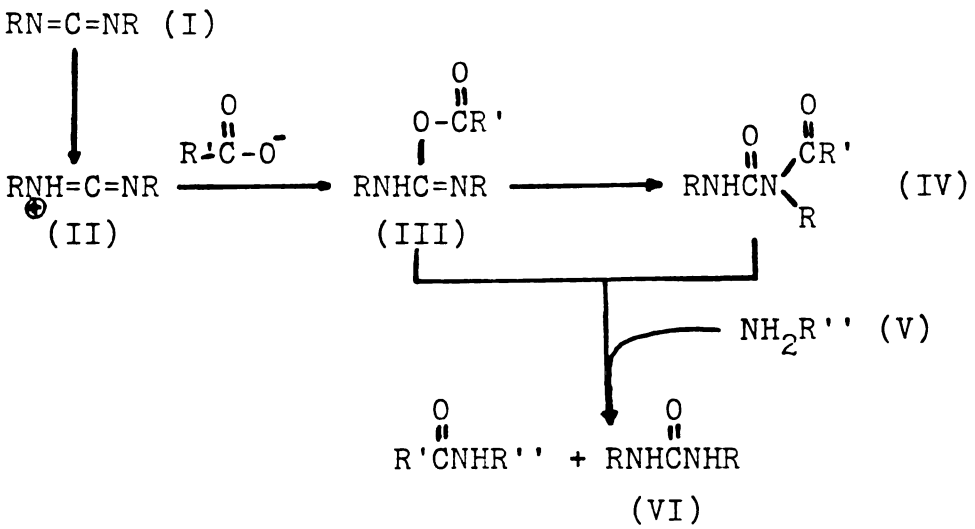


FIGURE 4 THE AFFINITY GEL SPACER-ARM DERIVITIZATION REACTION

protonated (II in Figure 4) and was subsequently attacked by the terminal carboxylate function of the spacer arm to produce the o-acylurea (III, Figure 4). The o-acylurea could form N-acylurea (IV, Figure 4) by intramolecular rearrangement. Either the o-acylurea or the N-acylurea could readily react with an amino group (V, Figure 4) (the hydrazide group of succinic acid dihydrazide in this case) to form a peptide type linkage with the release of an urea derivative (VI, Figure 4). In the study, succinyl dihydrazide was used instead of the simpler hydrazine molecule because the coupling reaction between the latter and the affinity gel spacer arm did not occur well when the reaction was attempted.

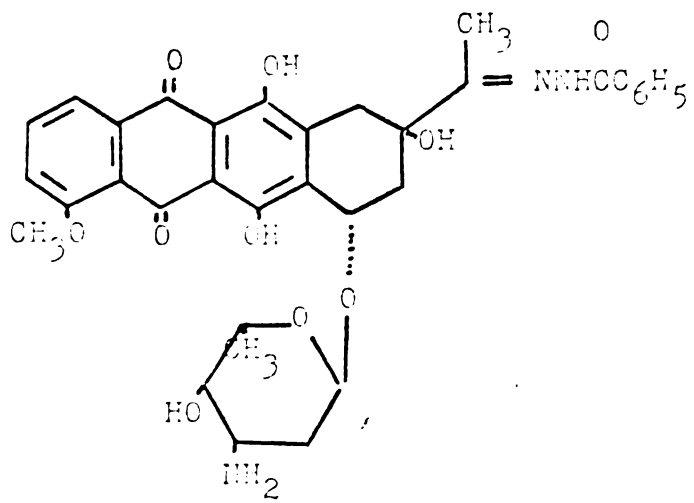
After washing away the unreacted materials and the urea product resulted from the reaction, the drug (either daunorubicin or adriamycin) was added to the affinity gel now with a spacer arm terminating in a hydrazide function. The C-13 carboxyl of the drug, the only functional group in the drug molecule capable of undergoing the reaction under the conditions, would react with the hydrazide to form a covalent linkage with the simultaneous elimination of a water molecule (163) (Figure 3, step 2). Finally, the affinity gel with the ligand attached was treated with acetone so that the unreacted hydrazide moieties that might be present could be blocked to avoid non-specific charges in the affinity gel (Figure 3, step 3).

As the result of these reactions, the ligand daunorubicin or adriamycin is anchored to the Affi-gel-202 matrix through a spacer arm of twenty-four atoms. The linkage between the drug molecule and the spacer arm thus formed resembles an active anthracycline antibiotic,

a rubidazone-like analog. Rubidazone (Figure 5) is an anthracycline antineoplastic agent currently under clinical trials (164-167). Cardiotoxic effects similar to those induced by adriamycin and daunorubicin have been demonstrated with rubidazone administration (168-170). Hopefully, this mode of attachment of the drug molecule to the affinity gel does not abolish the cardiotoxicity of the agents and the affinity gel-drug complex will be useful in isolating materials from the heart that are related to the myocardial effects of the agents.

As controls, affinity gels with daunomycinone, an inactive metabolite of daunorubicin *in vivo* (see below), as ligand and also the affinity gel with the spacer arm only and no drug attached were synthesized. The method of synthesis of these gels was similar to that used for the active compounds. In the case of daunomycinone, the coupling of the ligand to the spacer arm was done in the organic solvent pyridine, instead of water since the solubility of daunomycinone in aqueous solution is extremely low. The solubility of daunomycinone in various organic solvents including dioxane, dimethylsulfoxide, dimethyl sulfate and acetic acid was tested prior to the affinity gel synthesis and the drug appeared to be most soluble in pyridine. In addition, a trial reaction between daunomycinone and succinyl dihydrazide in solution as followed by thin layer chromatography also indicated that pyridine was the best solvent system. In the actual coupling reaction, 13 mg of daunomycinone was found to have reacted by quantitating the amount of drug recovered after the reaction using spectrophotometry, yielding a ligand drug capacity of 6 umole/ml affinity gel.

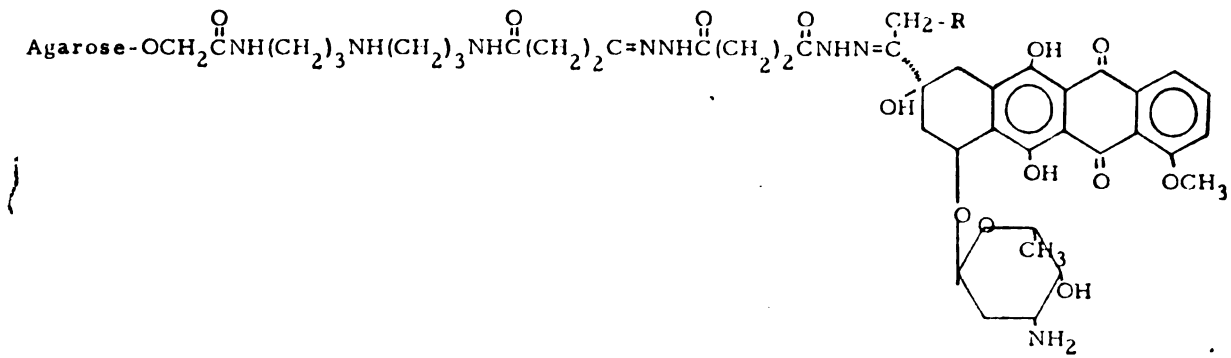
FIGURE 5



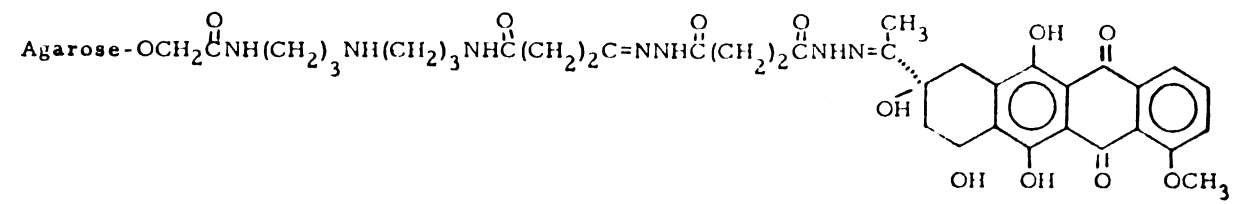
THE STRUCTURE OF RUBIDAZONE

Although adriamycin was readily soluble in aqueous solution, the coupling reaction between the drug and the affinity gel spacer arm did not proceed as well as it did in the case of daunorubicin. Even after nineteen days of shaking (see procedures, p. 34), only 7 μ mole of adriamycin reacted with each ml of the affinity gel while a ligand capacity of 10 μmole/ml gel was obtained for daunorubicin just after forty-eight hours of shaking. Apparently, the single replacement of the C-14 methyl (daunorubicin) by a methoxyl group (adriamycin) had a great deal of effect on the ligand-spacer arm coupling. The reason for this difference in reactivity is not known. The presence of an additional electronegative hydroxyl group adjacent to the C-13 carbonyl function in adriamycin should increase the electrophyllicity of this reactive group, thereby making it more prone to nucleophyllic attack by the hydrazide terminal of the spacer arm (171). However, the opposite effect was observed. One might hypohesize that the C-14 hydroxyl group in adriamycin may be imposing some steric influence on the C-13 carbonyl, making the hydrazide nucleophyllic attack on the latter more difficult. (See Figure 6 for the structures of the affinity gels.)

(1) Affinity Gels of the Active Drugs



(2) Daunomycinone Affinity Gel.



(3) Control Gel with Side-arm Only.

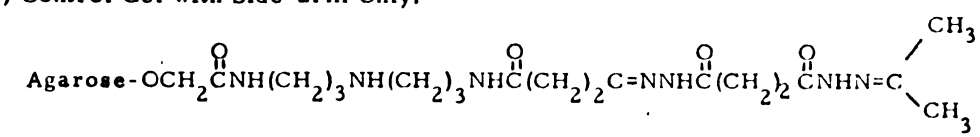


FIGURE 6 THE STRUCTURES OF THE AFFINITY GELS

C) THE DEVELOPMENT OF THE ISOLATION PROCEDURES

The second step of the study was to devise the methods for the isolation of any available non-nucleic acid substances from the heart that might recognize the anthracycline antibiotics. The procedures were developed with three major goals in mind:

(A) Since the drugs are extremely expensive and only difficultly available chemicals, it was very important to develop a method that used a very small amount of the drugs. This precluded the utilization of most conventional column techniques which were used in the majority of the affinity chromatographic procedures. Synthesizing anthracycline affinity gels for full sized columns, involved a time and cost that was prohibitive. Such large column procedures also usually have the problem of flow impedance after a few times of usage, especially when one is dealing with tissue homogenates. It seemed more desirable to develop an analytical procedure which only requires a small amount of affinity gel in the beginning and if the result was positive, a larger column can then be developed, if necessary.

(B) The second consideration was that the procedures devised would only isolate substances with high affinity towards the drug ligand. Since the anthracyclines have a sugar with an ionizable amino function which is charged at physiological pH, it was expected that the drug molecules will act as ionic exchangers and pick up negatively charged molecules non-specifically. Indeed, the anthracyclines have been shown to interact with a number of

anionic materials *in vitro* (63-70) (see p. 13). The isolation procedures needed to be able to screen out weak, non-specific binding species so that only substances with high affinity towards the drug molecule would be isolated.

(C) The procedures should be very gentle in nature so that the materials isolated will remain in their native state as much as possible. This is very important in the proper identification of the materials both physically and chemically at later stages.

The resulting procedures are summarized in Figure 7. The affinity gel (e.g. Daunorubicin affinity gel) was washed 25-30 times with pH 7, 0.01 M KH_2PO_4 buffer just before use (3 ml of buffer per wash with centrifugation sedimentation of the gel at the end of each wash). The thoroughly washed affinity gel was then incubated with heart homogenate from BFD_1 male mice (see section below for detailed method of homogenate preparation), treated extensively with deoxyribonuclease I and ribonuclease A, for fifteen minutes at room temperature on a reciprocal shaker. The resulting mixture of enzyme-treated homogenate and affinity gel was layered carefully on 8 ml of 60% sucrose at 4° C in a 10 ml conical centrifuge tube at the end of the incubation period. The mixture was centrifuged at 2,500 rpm for 15 minutes at 4° C in an International Centrifuge (refrigerated model). At this time, the affinity gel was sedimented to the bottom of the centrifuge tube while the rest of the homogenate remained at the top of the sucrose solution. The majority of the sucrose was then removed with the homogenate using a long tipped Pasteur pipet. The remaining gel

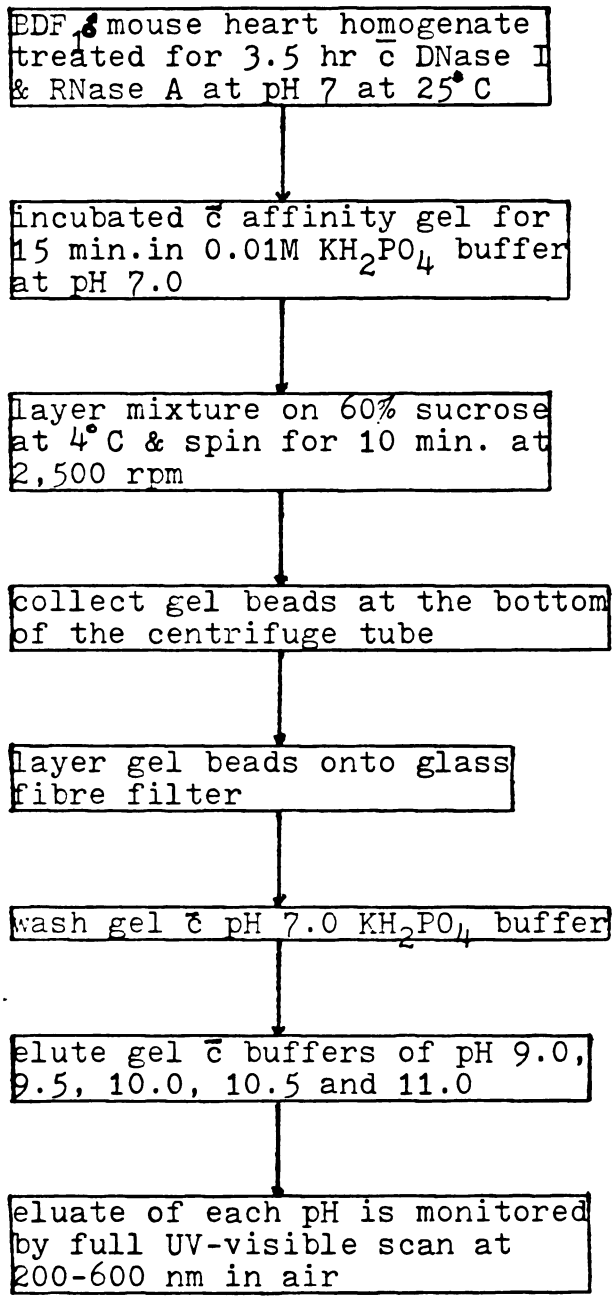


FIGURE 7

Procedures for the Isolation of Drug Receptive Materials

beads were transferred onto a glass filter (Buchner funnel with fritted disc. Corning #36060, medium grade), washed with two ml of 60% sucrose solution and drained with vacuum. The affinity gel was then washed with two ml of 0.01 M KH_2PO_4 buffer (pH 7) and followed by a batch pH step elution procedure. After preliminary study of the best range, a pH elution was done sequentially using 0.04 M glycine buffers at pH 9.0, 9.5, 10.0, 10.5 and 0.05 M Na_2HPO_4 buffer at pH 11.0. (The gel beads were soaked in 0.5 ml of the elution buffer for two minutes before the buffer was drained by vacuum. The process was repeated three more times to complete the elution at each pH step. Consequently, a total of 2 ml of buffer was used for each step of the pH elution.) The eluates at each pH were pooled and subjected to U.V.-visible absorption spectrophotometric analysis.

The greatest advantage of this procedure is that it not only selected for high affinity ligand-binding substances, but required a minimum amount of affinity gels. As little as 0.2 ml of the affinity gel was needed for each experiment. Since the gel-anthracycline linkage is not very stable at very high pHs, the ligand starts to dissociate from the gel matrix when the gel is eluted with buffers of higher pH. Therefore, the affinity gel was only used for one experiment to assure reliability of the procedure and this makes the small amount of affinity gel needed per experiment an even more important advantage. The procedures can be carried out fairly easily with no need for expensive and sophisticated equipment. The spinning of the affinity gel through 60% sucrose serves two functions. Firstly,

the affinity gel can be separated from the rest of the homogenate through this simple, brief centrifugation step, hopefully bringing some components from the heart homogenate that have an affinity towards it to the bottom of the centrifuge tube. Secondly, this centrifugation step serves the purpose of screening out weakly interacting species. Materials that have weak affinity towards the affinity gel are likely to detach from the gel when it is sedimenting through the dense 60% sucrose solution at 4° C. Consequently, only substances with relatively high affinity towards the affinity gel will survive this step and settle to the bottom of the centrifuge tube with the gel particles. One major value of this method is that species from the heart homogenate interacting with the affinity gel weakly or non-specifically hydrophobically will probably be released as the affinity gel is settling through the extremely hydrophobic sucrose solution, thus only the very high affinity binding substances are retained on the drug ligand.

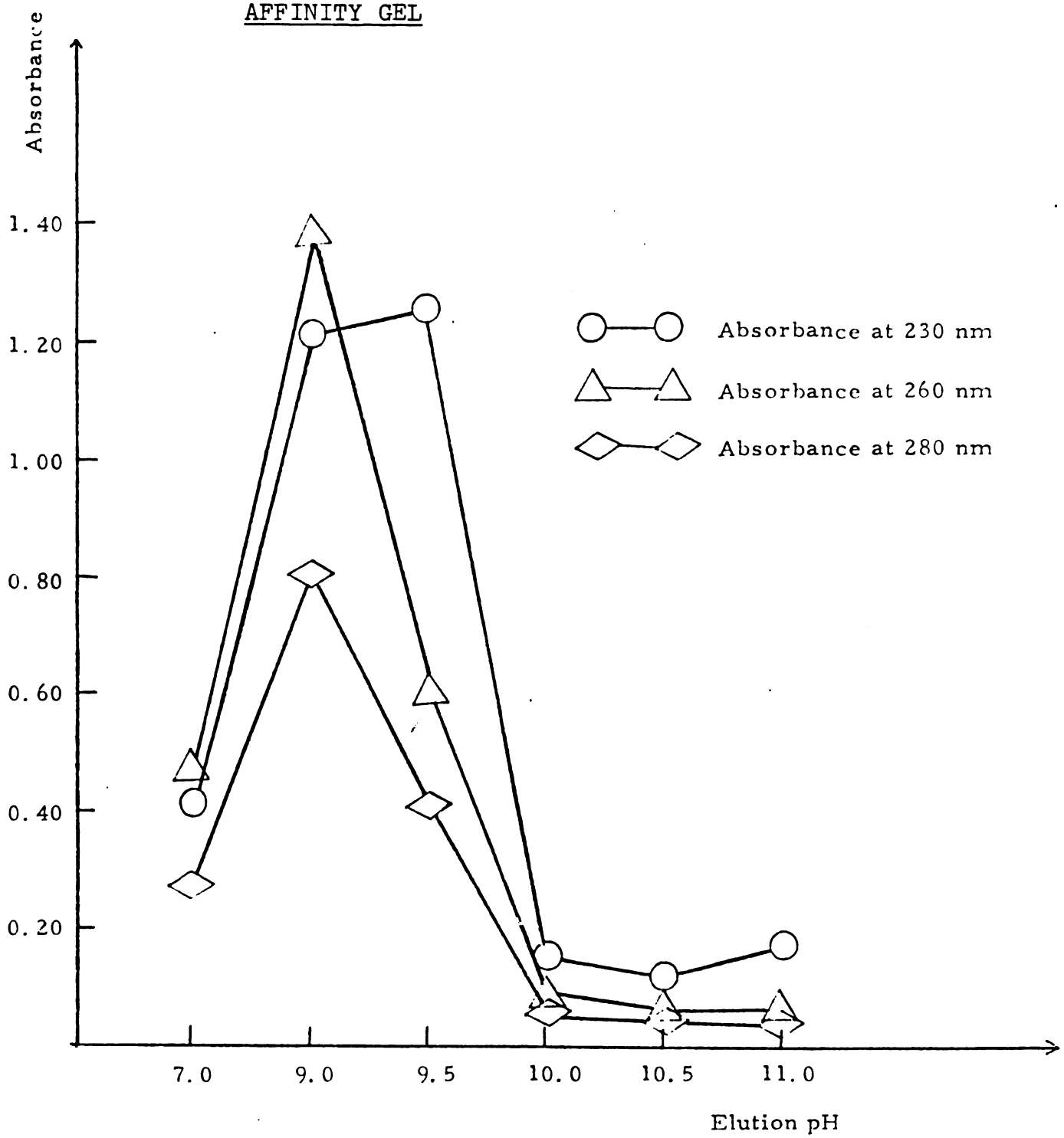
D) PREPARATION OF THE MOUSE HEART HOMOGENATE

Four to five mouse hearts (BDF_1 male mice at 20-25 gm, the total heart tissue weight was approximately 1 gm) were excised from mice freshly sacrificed by cervical dislocation, and finely minced on ice with a razor blade. The tissue was rinsed with cold distilled water to remove the blood and transferred to a potter homogenizer following removal of visible clots. Four ml of phosphate buffer (0.01 M KH_2PO_4 at pH 7.0) at 4° C was then added. While on ice, the tissue was homogenized with 150 passes over approximately ten minutes. The homogenate was filtered through cheese cloth into a 10 ml graduated cylinder, and the final volume brought to 5 ml with the addition of more phosphate buffer at 4° C. The homogenate was then treated with 0.5 mg of deoxyribonuclease I (Sigma Chemical Company, Catalog #DN-CL from bovine pancreas) and 1.0 mg of ribonuclease A (Sigma Chemical Company, Catalog #R-5152 Type III-A from bovine pancreas) at room temperature for three and a half hours.

E) ISOLATION OF NON-NUCLEIC ACID RECEPTIVE MATERIALS FROM THE MOUSE HEART USING DAUNORUBICIN AFFINITY GEL

Daunorubicin affinity gel was incubated with deoxyribonuclease I and ribonuclease A treated mouse heart homogenate for fifteen minutes and then subjected to the pH elution procedures as outlined on p. 43. Figure 8 shows the pH elution profile of the affinity gel resulting from such an experiment. A large amount of material was eluted from the daunoubicin affinity gel after the gel had been incubated with the heart homogenate the majority of which was eluted at pH 9.0-9.5. The materials eluted at pH 9.0 had an absorption peak at approximately 260 nm while the materials that came off at pH 9.5 had strong absorption in the 200-240 nm region. Very little material came off the gel at the higher pHs (pH 10-11). At pHs above 10.5, the drug ligand began to slightly dissociate from the affinity gel matrix as evidenced by the appearance of a very light red color in the eluates. It is therefore not likely that components binding to the drug molecule will remain tightly attached to the affinity gel at these pHs. One can be reasonably sure that all of the materials from the heart homogenate with affinity towards the ligand daunorubicin will be eluted by this procedure.

FIGURE 8 THE pH ELUTION PROFILE OF THE DAUNORUBICIN AFFINITY GEL



F) CONTROL AFFINITY GEL WITH NO DRUG ATTACHED AND DAUNOMYCINONE LIGAND AFFINITY GEL

It was very encouraging that materials could be isolated from the mouse heart using the daunorubicin affinity gel isolation procedures. However, before jumping to any premature conclusions, a very important question had to be considered: Do the substances eluted from the daunorubicin affinity gel at pH 9.0 and 9.5 after the gel has been incubated with nuclease-treated mouse heart homogenate really represent materials with selective affinity to the ligand molecule? Four possibilities were considered.

(A) The materials eluted are binding to the gel components, e.g. agarose, spacer arm.

(B) The materials eluted are recognizing the spacer-arm-linkage structure.

(C) The materials eluted represent components with affinity towards daunorubicin.

(D) Any combinations of the above possibilities.

Different approaches can be used to distinguish these possibilities. An obvious one is to synthesize affinity gel without the drug ligand and investigate the behaviour of this affinity gel in the same system. However, this control gel lacks the ligand-spacer arm linkage area in the daunorubicin affinity gel and will not tackle hypothesis number two. To solve this problem, daunomycinone affinity gel was

synthesized in addition to the control gel with the spacer arm only.

In vitro and *in vivo* studies have shown that both daunorubicin and adriamycin are metabolized extensively by various tissues (172-176). The two mammalian enzyme systems responsible for the metabolism of the compounds are the cytoplasmic aldo-keto reductase which appears to be ubiquitous in its distribution (177-178) and the microsome associated glycosidases (179-182). The enzymic products of the aldo-keto reductase of daunorubicin and adriamycin in which the C-13 carbonyl of the drugs is reduced to a hydroxyl function, daunorubicinol and adriamycinol respectively, retain inhibitory activity against both DNA and RNA synthesis in cells although the activity is lower than the parent compounds (183-184). On the other hand, the glycosidases are believed to inactivate the anthracyclines by releasing the aglycone products (185-187). Although both enzyme systems metabolized the drugs readily *in vitro*, *in vivo* experiments showed that the C-13 reduction products were by far the major metabolites in tissues while high aglycone levels were only found in the liver, the kidney, the spleen and the small intestine (175-176). Daunomycinone, one of the aglycone metabolites of daunorubicin (175-176,179), lacks any significant activity as an antineoplastic agent (188) and was shown not to interact with DNA *in vitro* (189). There has been some controversy concerning the possible cardiotoxic action of daunomycinone. Herman and his co-workers reported that hamsters treated with daunorubicin or adriamycin had high levels of aglycones in heart and liver tissues (190). However, the contrary was observed in other animal species such as the rat and the rabbit (175-176).

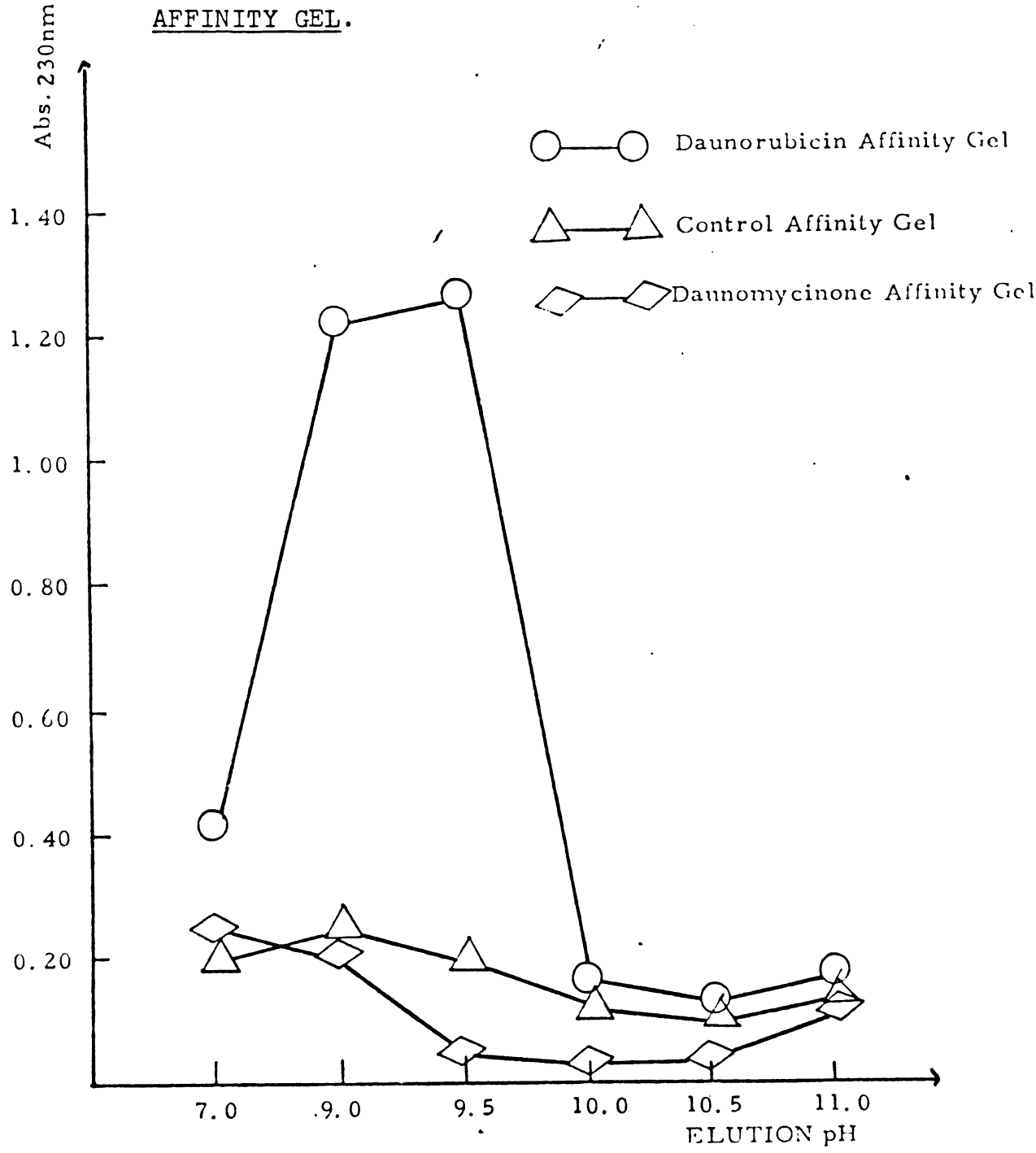
Puzzled by these observations, Bachur and his colleagues attempted to reproduce Herman's data by repeating his experiment; they were only able to find low levels of aglycones in the heart and in the lung of the hamsters they used in their study (173). In another attempt to link daunomycinone with cardiotoxicity, Herman's group reported that the drug caused an immediate increase in the coronary perfusion pressure in perfused isolated dog hearts whereas a slower and gradual increase was seen when either daunorubicin or adriamycin was used (191). Moreover, N-acetyl-daunomycin, an analog that was shown to be metabolized to the aglycone product to a much lesser extent, did not show such alteration in perfusion pressure. Based on these results, the authors suggested that daunomycinone, instead of the parent drug, daunorubicin, was responsible for the induced increase in the coronary tone. These data are disputable, since the daunomycinone in the study was dissolved in dimethyl sulfoxide instead of the physiological saline used for daunorubicin and no vehicle control was done. In contrast to Herman's viewpoint, Adamson proposed that the cardiotoxicity of the anthracyclines resided in the daunosamine portion of the molecule while the anti-tumor activity required the complete molecule (192).

Regardless, the daunomycinone affinity gel is a good control since it contains almost all the components present in the daunorubicin affinity gel: the agarose gel matrix, the spacer arm, the succinyl dihydrazide extension of the spacer arm, the spacer arm-ligand linkage area and the daunorubicin aglycone chromophore. The only thing that is missing in the daunomycinone affinity gel is the

amino sugar, daunosamine, which according to Adamson, is an essential part of the molecule that causes the cardiotoxic effect.

Figure 9 illustrates the results of experiments using the different affinity gels synthesized. The circles represent the pH elution profile of daunorubicin affinity gel after the gel has been incubated with nuclease-treated heart homogenate. As mentioned before, materials were eluted at pH 9.0 and 9.5 that could be detected by U.V.-visible spectrophotometry. In sharp contrast, very little material was washed off from either the control gel with no drug attached or the daunomycinone affinity gel in spite of the fact that these gels had been put through identical isolation procedures. Therefore, it is reasonable to conclude that the materials eluted off the daunorubicin affinity gel at pHs 9.0 and 9.5 do represent constituents from the heart that have an affinity towards the daunorubicin ligand and not the gel components. Furthermore, these substances appear to recognize the complete molecule of daunorubicin. The chromophore of the drug alone is not sufficient to pull these materials from the heart homogenate.

FIGURE 9 THE pH ELUTION PROFILES FOR THE CONTROL GEL, THE DAUNORUBICIN GEL AND THE DAUNOMYCINONE AFFINITY GEL.

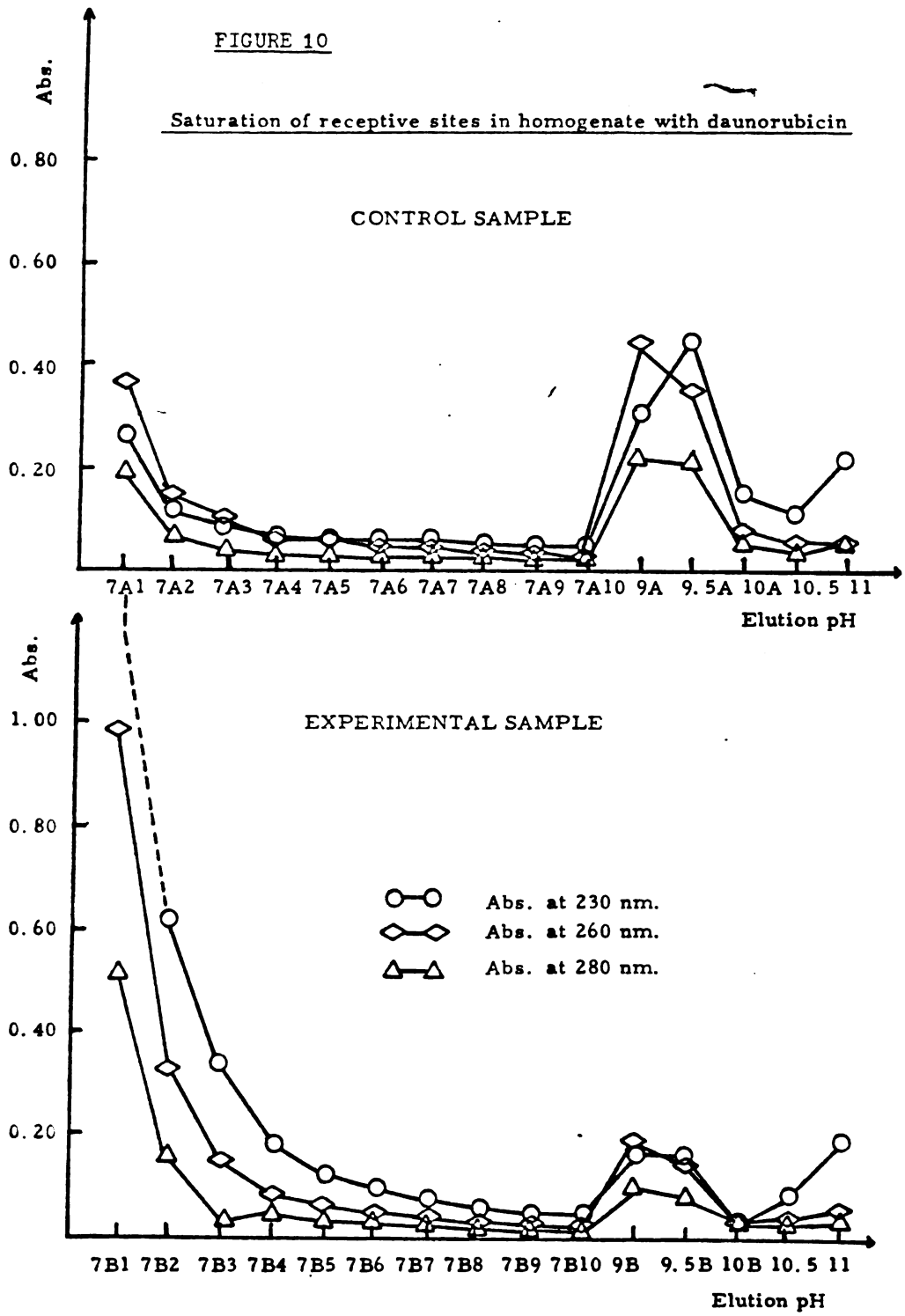


G) SATURATION OF THE BINDING SITES IN THE HEART HOMOGENATE BY DRUG PRETREATMENT

If the pHs 9.0 and 9.5 eluted materials from the daunorubicin affinity gel are indeed recognizing the ligand molecule, it should be possible to saturate the binding sites in the heart homogenate by pretreating the homogenate with daunorubicin prior to the incubation of the affinity gel with the homogenate. This hypothesis was subjected to testing by the following experiment: after the nucleases treatment of the heart homogenate, 20 mg of daunorubicin HCl was added to the homogenate (final concentration of daunorubicin 8 mg/ml, 14 mM) before the daunorubicin affinity gel was incubated with it. The affinity gel was then separated from the homogenate and eluted with buffers in the usual manner. Figure 10 shows the results of this experiment. Since the added daunorubicin adsorbed to the affinity gel to some extent, after the daunorubicin affinity gel was spun down the 60% sucrose solution and placed on a glass filter (see Figure 7), it had to be washed with 0.01 M KH_2PO_4 buffer to clean off the adsorbed drug before the pH elution step could be carried out. (The pH 7 washes are depicted as 7As or 7Bs on the graph of Figure 10. Each pH 7 wash fraction had to a volume of 2 ml.) The top panel (control sample) shows the data obtained from a parallel sample of daunorubicin affinity gel that had been put through the same procedures as the experimental sample except the homogenate was not pretreated by daunorubicin. We see that even after extensive pH 7 washing of the affinity gel (7A1-7A10 total 20 ml washes), the daunorubicin receptive materials

FIGURE 10

Saturation of receptive sites in homogenate with daunorubicin



from the heart continued to bind tightly to the gel. As soon as the elution pH was raised to pH 9 and 9.5, these materials immediately dissociated. In contrast, a much smaller amount of materials could be eluted from the affinity gel incubated with homogenate pretreated with daunorubicin (bottom panel). Although this experiment by itself does not show unequivocally that the daunorubicin added to the homogenate prevents the receptive materials from getting on the affinity gel by saturating the binding sites in the homogenate, in combination with other data, it further supports the conclusion arrived at from the previous experiments, that the heart materials are indeed recognizing the daunorubicin molecule. A more careful study on the ability of free drug to elute these cardiac materials from the affinity gel would provide a more convincing argument. These experiments were done and will be discussed in a later section.

H) SIZE DISTRIBUTION OF DAUNORUBICIN AFFINITY MATERIALS

In an attempt to get an idea of the composition of the pHs 9.0 and 9.5 eluates of the daunorubicin affinity gel after the gel has been incubated with heart homogenate, these fractions were subjected to Sephadex gel filtration for sizing. The samples, which have been lyophilized and stored dessicated in the freezer until the procedure, were dissolved in 0.05 ml of pH 7 0.01 M KH_2PO_4 buffer and applied to the Sephadex column (Sephadex G-75 fine, Pharmacia Chemical Inc., Catalog #7571). The samples were eluted with the same buffer. Eight drops were collected in each fraction at a flow rate of approximately three drops per minute. The collected fractions were then subjected to ultraviolet spectrophotometric analysis (full UV scan from 200-350 nm for each fraction). Figure 11 shows the Sephadex G-75 elution profile of the pH 9.0 eluted materials. Apparently, the eluate was composed of two components: a high molecular weight component with an absorption peak at approximately 275 nm and strong absorption in the 200-230 nm region which came out in the void volume of the column; and a low molecular weight component with an absorption peak at approximately 256 nm which came out in about the bed volume of the column.

The Sephadex G-75 elution pattern (Figure 12) of the pH 9.5 eluate is very similar to that obtained for the pH 9.0 eluate. Again, we see two components: a high molecular weight one and a low molecular weight one with very similar Sephadex chromatographic and spectrophotometric absorption properties to the corresponding parts in the pH 9.0 eluted

FIGURE 11

Sephadex G-75 Elution Profile of the pH 9 Eluted Materials.

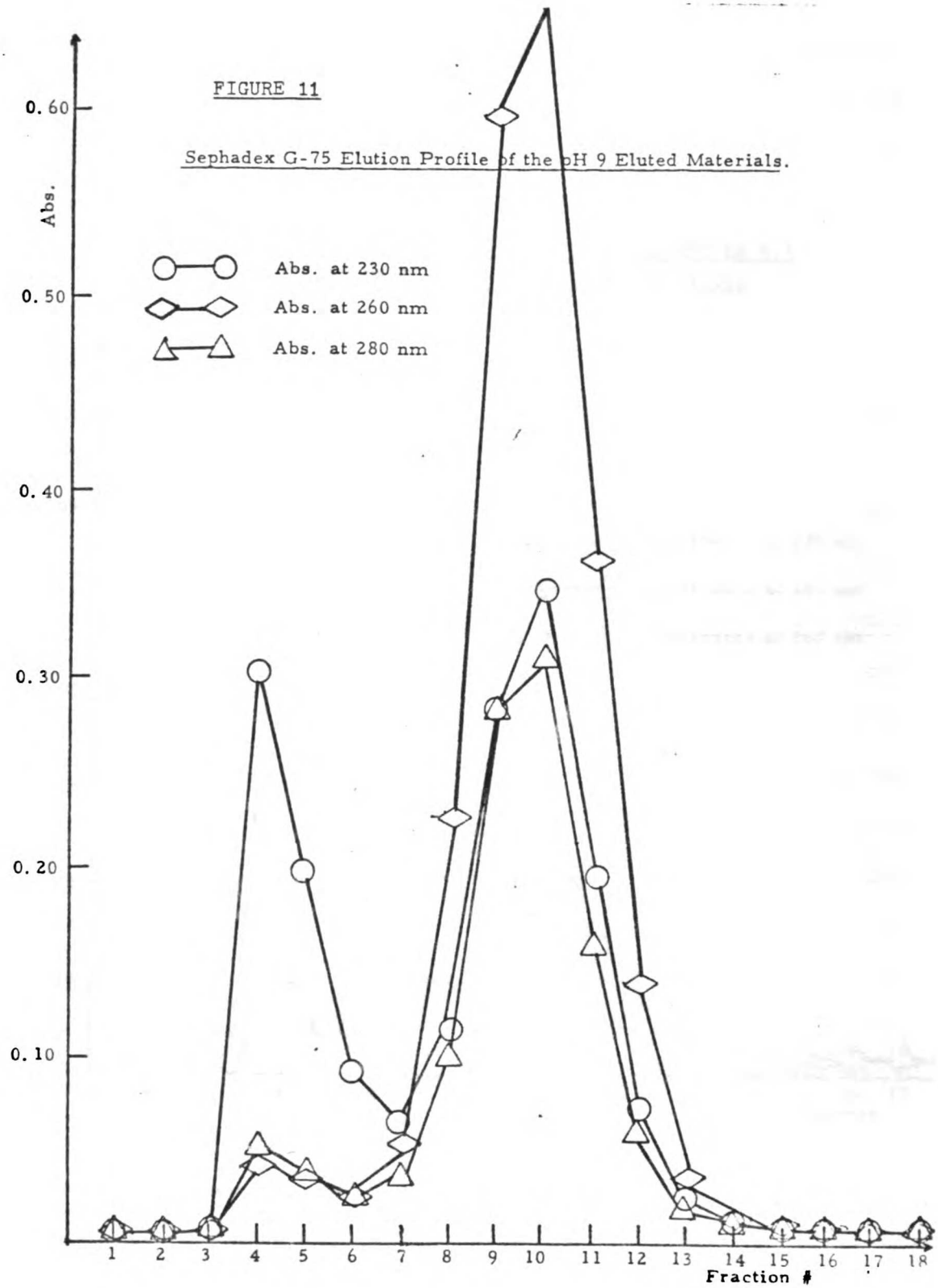
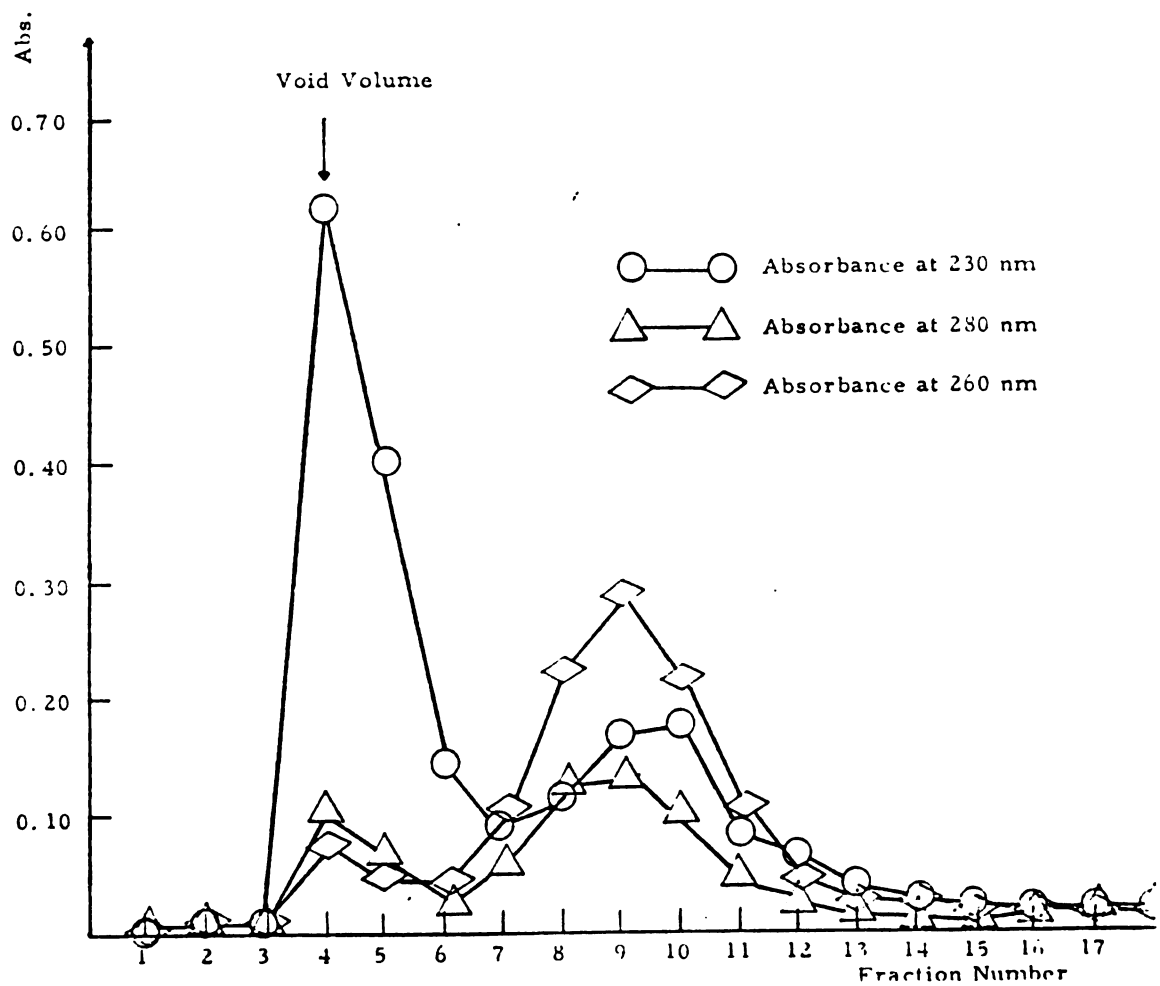


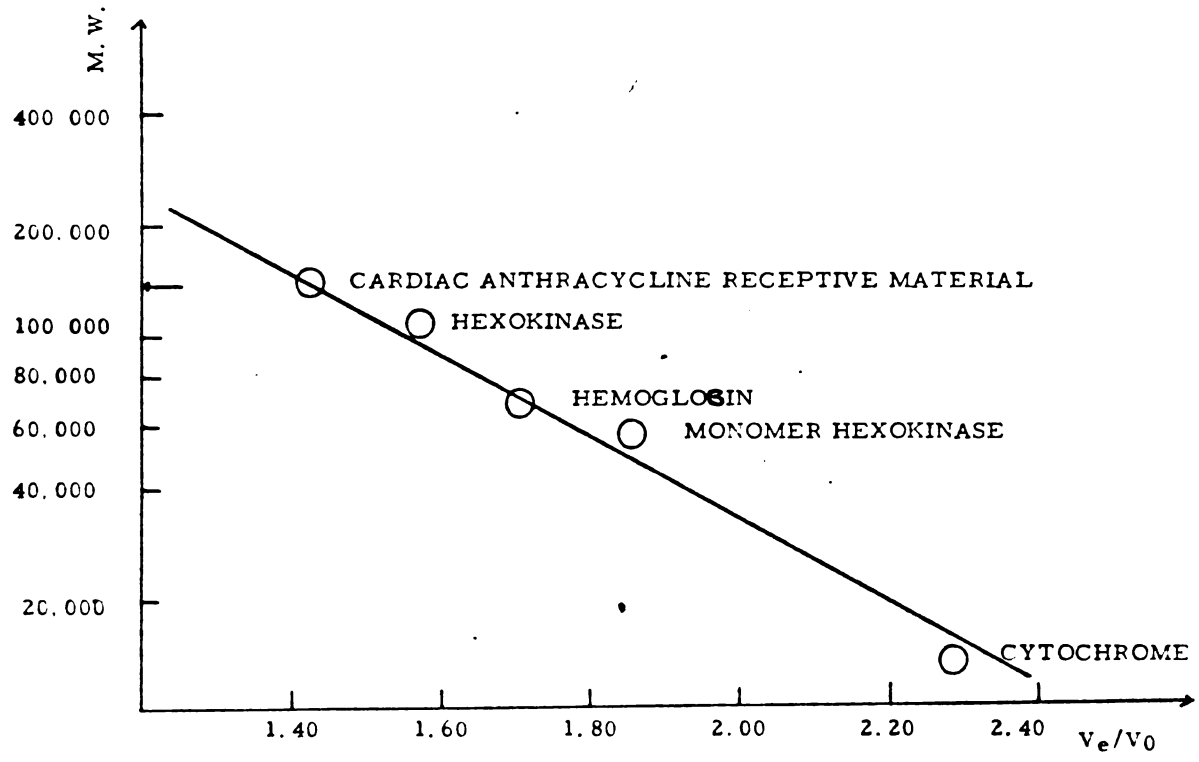
FIGURE 12 SEPHADEX G-75 ELUTION PROFILE OF THE pH 9.5 ELUATE OF THE DAUNORUBICIN AFFINITY GEL



materials. The majority of the low molecular weight substance appears to be present in the pH 9.0 eluate while most of the high molecular weight component is found in the pH 9.5 eluted fraction. From the UV absorption spectrum and other observations, the low molecular weight material appears to have properties consistent with that of some nucleotide derivative. It could represent a nucleotide coenzyme or the digestive products of the nucleases treatment carried through the isolation procedures. Since the molecular exclusion limit for the Sephadex G-75 gel filtration system is approximately 75,000 daltons (for globular proteins), anything with a molecular weight higher than that will probably be eluted in the void volume of the column. To obtain a molecular weight estimation of the high molecular weight component in the pH eluates, one has to choose a gel system with a higher exclusion limit. Sephadex G-200 fine particle gel filtration was used for this purpose (Sigma Company, G-200-120). Fraction 4-6 obtained from the Sephadex G-75 chromatography of the pH 9.5 eluted materials were pooled and lyophilized. The dried material was redissolved in 0.1 ml of the pH 7 phosphate buffer and applied to the column. The sample was eluted in the same buffer (0.01 M KH_2PO_4 pH 7.0). Six drops were collected in each fraction at a flow rate of approximately 2.5 drops per minute. The collected fractions were subjected to UV scan from 200-350 nm. Figure 13 represents a molecular weight calibration curve obtained from this experiment. As can be seen from the figure, the high molecular weight daunorubicin cardiac receptive material falls on a position corresponding to an apparent molecular weight of approximately 140,000

FIGURE 13

Sephadex G-200 Molecular Weight Estimation of the Cardiac Receptive Material for Daunomycin.

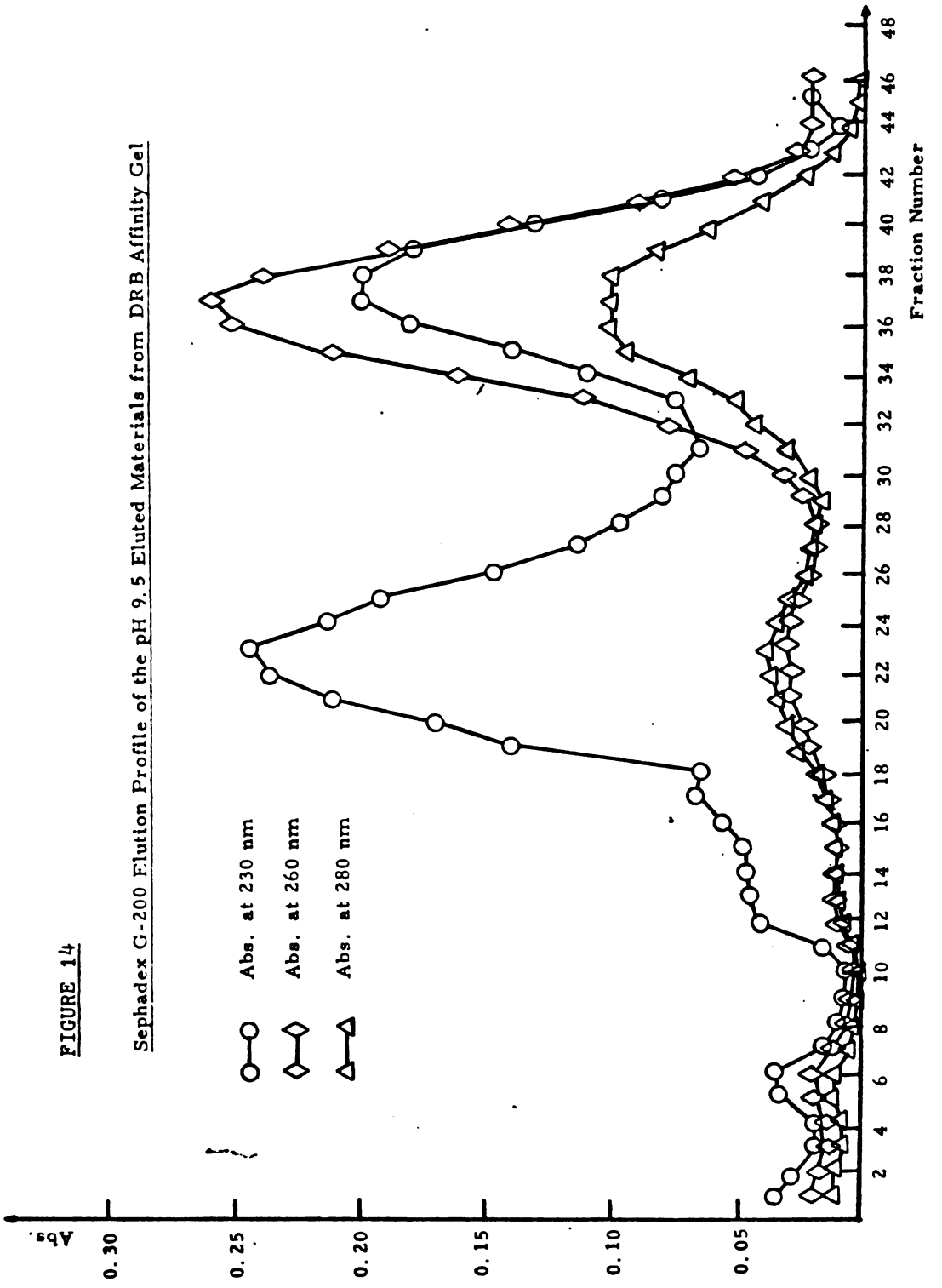


daltons.

Since the amount of materials obtained and used in these chromatographic experiments was very small, small Sephadex columns had to be used to prevent excessive dilution (10 ml pipets were modified for this purpose). In another experiment, pH 9.5 eluates from daunorubicin affinity gel after incubation with heart homogenate were pooled from several isolations and applied to a larger G-200 Sephadex column (total volume 26 ml). The elution profile obtained (Figure 14) again showed the now familiar pattern: a high molecular weight component eluted at approximately 145,000 daltons and a low molecular weight component eluted at very late fractions (ATP was eluted at fraction #38 in this column). So the data obtained from the small columns are reasonably accurate.

FIGURE 14

Sephadex G-200 Elution Profile of the pH 9.5 Eluted Materials from DRB Affinity Gel

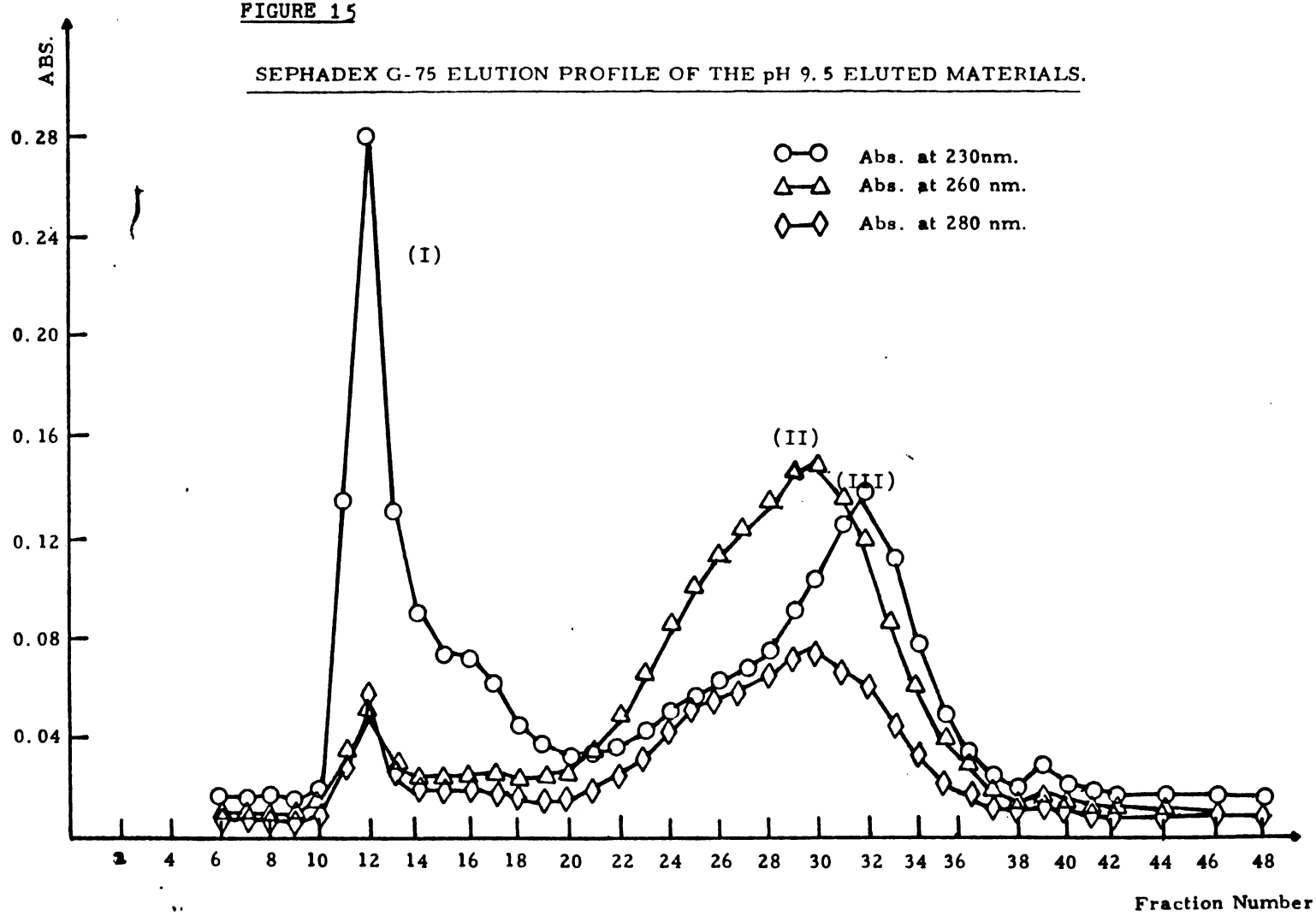


I) PROTEASE TREATMENT OF THE pH 9.5 ELUTED MATERIALS FROM THE DAUNORUBICIN AFFINITY GEL

The high molecular weight component of the pH 9.5 eluted materials has an UV absorption spectrum suggestive of a protein nature. Consequently, experiments were performed to investigate the Sephadex elution behavior of this material in the presence of protease. The lyophilized materials eluted from a daunorubicin affinity gel at pH 9.5 after the gel has been incubated with nuclease-treated mouse heart homogenate were redissolved in 0.3 ml of 0.01 M KH_2PO_4 buffer at pH 7.0. 0.2 ml of this solution was removed and placed in a test tube containing 0.15 mg of protease (Sigma Chemical Company catalog #P-5130. Isolated and purified from Streptomyces griseus. Type VI). The mixture was then incubated at room temperature for 24 hr at which time 0.1 ml of the sample was loaded on a Sephadex G-75 column and eluted with phosphate buffer at pH 7.0 (0.01 M KH_2PO_4). As control, a parallel sample of the pH 9.5 eluted materials without the protease treatment was applied to the G-75 column after the sample had been at room temperature for 24 hours, to obtain an elution profile for comparison (Figure 15). With the use of a larger Sephadex column and improved chromatographic techniques, one noticed that the "low molecular weight daunorubicin receptive material" seen before could actually be further resolved into two parts. The component with an absorption peak at 260 nm which was believed to be a nucleotide coenzyme or a nuclease-degradative product was eluted slightly before the bed volume (peak II in Figure 15) and another component with strong absorption at

FIGURE 15

SEPHADEX G-75 ELUTION PROFILE OF THE pH 9.5 ELUTED MATERIALS.

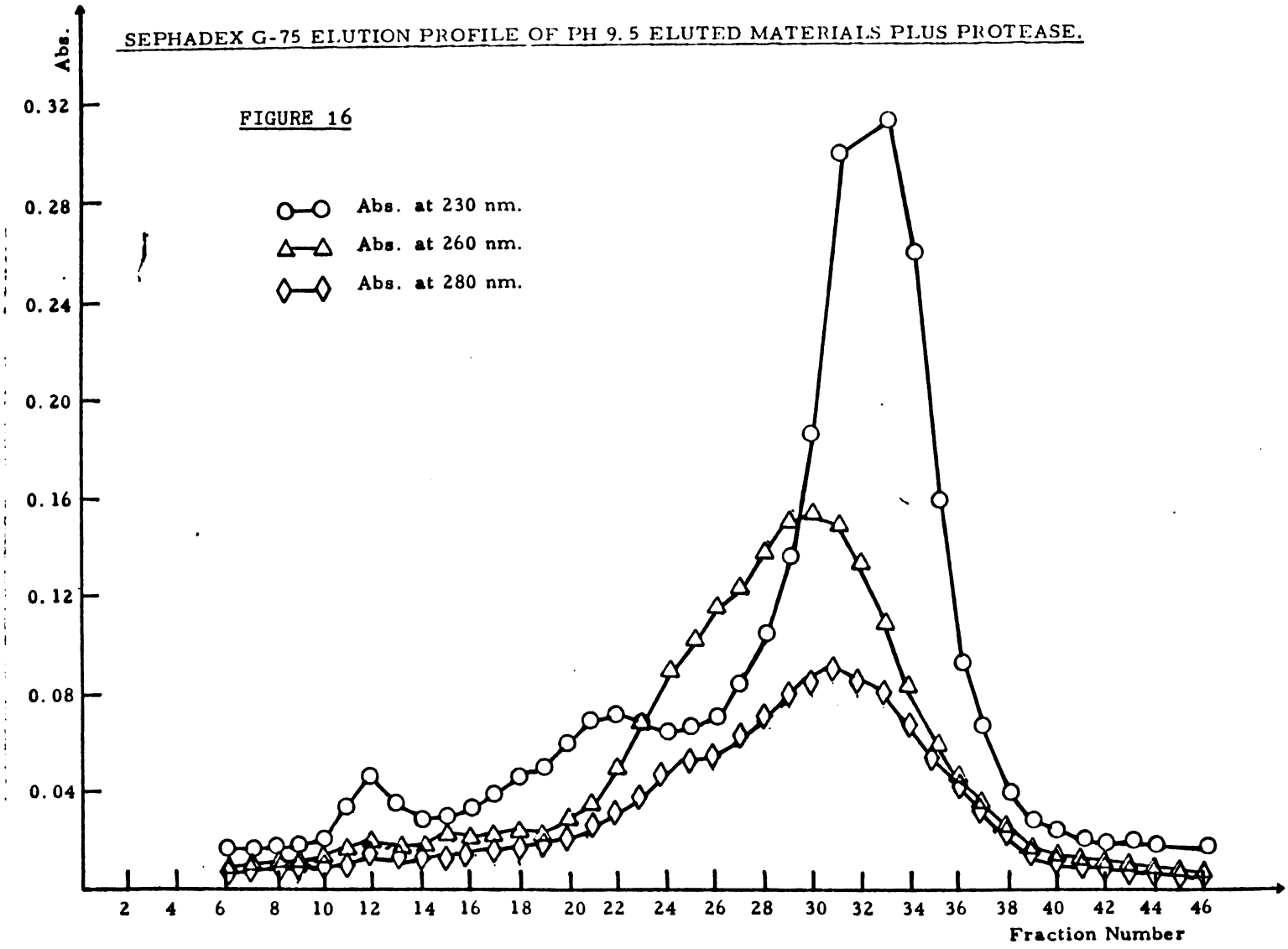


230 nm was eluted in the bed volume (peak III in Figure 15). If the daunorubicin affinity gel was put through the pH elution procedures without being incubated with the mouse heart homogenate, one would still detect this last component in the pH 9.5 eluate as demonstrated by G-75 Sephadex chromatography. Furthermore, this component (as identified by its spectrophotometric absorption and Sephadex G-75 chromatographic properties) was present in all the daunorubicin affinity gel eluates at higher pHs (pHs 10-11) with increasing amounts in the more basic eluates. Therefore, it was concluded that this material in the pH 9.5 eluate (peak III in Figure 15) derived from the affinity gel as the result of the pH elution and was not a constituent of the heart homogenate. The high molecular weight daunorubicin receptive material was eluted in the void volume of the Sephadex column as usual (peak I in Figure 15).

With the treatment of the bacterial protease, the high molecular weight receptive material peak disappeared while a new species with strong absorption at 230 nm emerged in the bed volume (Figure 16). This new peak stained intensively with the ninhydrin reaction, under conditions indicating the presence of Alpha amino compounds in this peak (193). In contrast, the low molecular weight receptive material (peak II in Figure 15) was not affected by the protease treatment to any significant extent.

SEPHADEX G-75 ELUTION PROFILE OF PH 9.5 ELUTED MATERIALS PLUS PROTEASE.

FIGURE 16



J) SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE HIGH MOLECULAR WEIGHT DAUNORUBICIN RECEPTIVE MATERIAL

A number of proteins if bound by the anthracyclines in the heart could be logical receptors. A simple way to eliminate dozens of candidates at once is to accurately assess the properties of the one isolated and compare with those of the candidate materials.

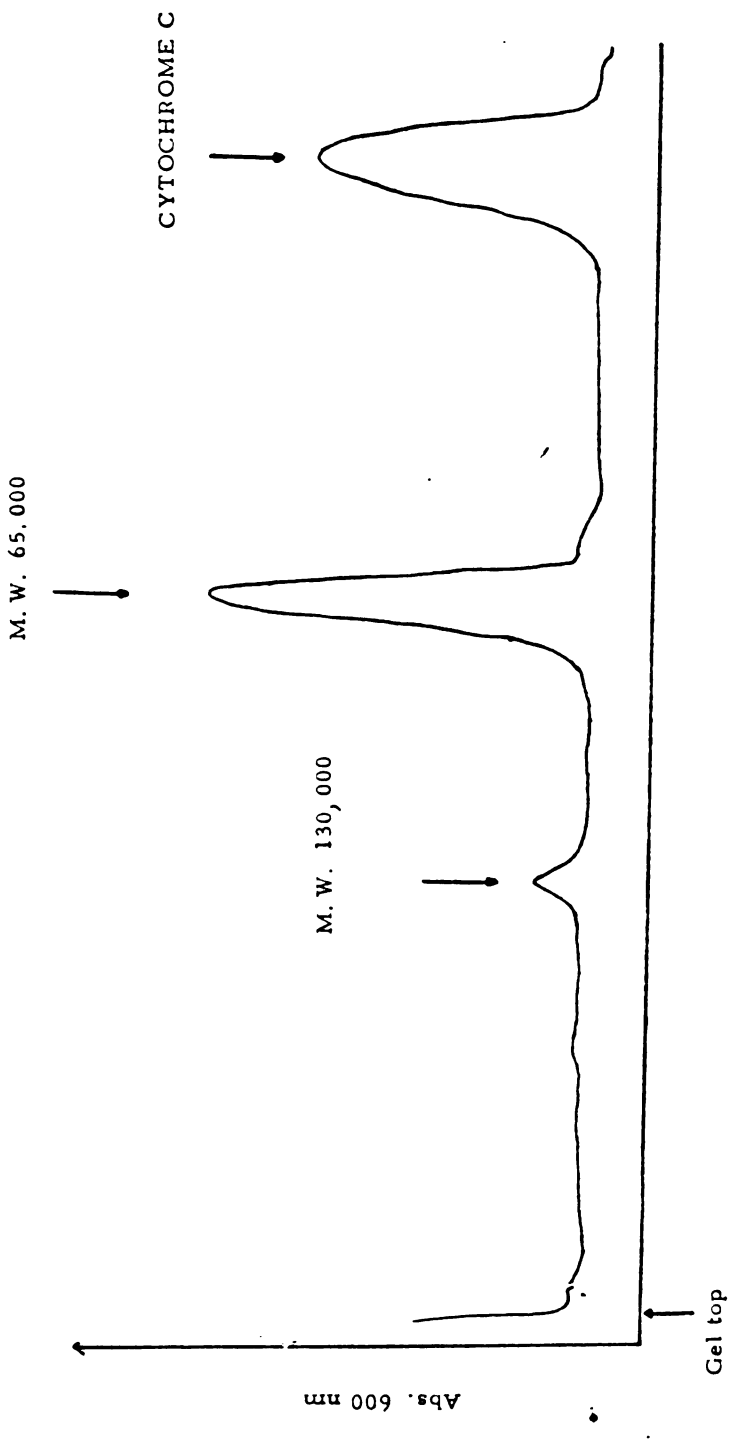
SDS-polyacrylamide gel electrophoresis was utilized to analyze the subunit structure of the protein receptive substance for daunorubicin isolated from the heart. Appropriate fractions of the Sephadex G-200 elution were pooled and lyophilized. The material was redissolved in solubilizing buffer (solubilizing buffer contained 0.04 M dithiothreitol, 0.5% sodium dodecyl sulfate, 1 mM EDTA and 20% sucrose in 1/10 running buffer; running buffer consisted of 0.205 M Tris HCl, 0.205 M acetic acid and 0.1% sodium dodecyl sulfate, pH 6.4). The protein solution was then boiled in water for five minutes before it was applied to the gel. The polyacrylamide gels used were pre-cast gels purchased from the BioRad Chemical Company (4% polyacrylamide precast gel catalog #161-1001). The procedures for the actual electrophoretic run of the samples are described in detail in BioRad technical bulletin #1038. A modified Fairbank method was used for the staining of the proteins (194). Four concentrations of Coomassie blue stain were used in the staining procedures (Coomassie brilliant blue R-250, BioRad Laboratories catalog #161-0400). The constitution of the staining solutions is as follows:

	Coomassie Blue	Isopropyl Alcohol	Acetic Acid
Fairbank #1	500 mg	250 ml	100 ml
Fairbank #2	50 mg	100 ml	100 ml
Fairbank #3	25 mg or less	-	100 ml
Fairbank #4	-	-	100 ml

* Make to a total volume of 1 litre with distilled water.

The gels were placed in Fairbank #1 stain immediately after the electrophoretic run. After 24 hours, they were transferred into stain #2 for 4 hours followed by another 4 hours incubation in stain #3. Complete destaining was accomplished by several changes in Fairbank #4.

Figure 17 represents the mobility profile of the daunorubicin cardiac receptive protein obtained in the pH 9.5 eluate. The material could be resolved into two peaks: one with an apparent molecular weight of approximately 130,000 and the other with a molecular weight of approximately 65,000. This profile did not depend on the presence of reducing agents like dithiothreitol. Increasing the concentration of SDS from 0.5% to 3% did not change the relative ratio of the two peaks. Thus the 140,000 daltons molecular weight peak in the G-200 Sephadex column appears to consist of two different proteins: a) a protein with a molecular weight of approximately 140,000 which is not dissociated by SDS treatment, and b) a dimer with approximate molecular weight of 65,000 per subunit that does not require dithiothreitol to be dissociated.



SDS-polyacrylamide gel electrophoresis mobility profile of the daunorubicin cardiac receptive material

FIGURE 17

From these simple biochemical analytical techniques (Sephadex gel permeation chromatography and SDS-polyacrylamide electrophoresis), one is able to eliminate a large number of potential receptive proteins for daunorubicin that may explain the observed cardiotoxic effects. For example, myoglobin (M.W. 16,900) and mitochondrial enzymes such as succinic dehydrogenase (M.W. 190,000) and alpha-ketoglutarate (particle wt. of the complex $\simeq 2 \times 10^6$) can be eliminated. Contractile proteins myosin (subunit molecular weight under SDS conditions used in the experiment = 200,000) and actin (molecular weight 42,000) which are anticipated to be two major proteins in the myocardial muscle cells can also be excluded. The observation that these proteins were not picked up by our affinity gel isolation procedures in spite of the report that they interact with daunorubicin in vitro (67) confirms our expectation that the receptive materials obtained with the methodology represent substances with relatively high affinity towards daunorubicin (see p. 45).

K) INTRACELLULAR LOCALIZATION OF THE DAUNORUBICIN RECEPTIVE MATERIALS

To further characterize the daunorubicin receptive materials obtained using our isolation procedures, experiments were performed to determine the intracellular locations of these substances. The mouse heart homogenate was prepared as described previously and then centrifuged at 100,000 xg for 60 minutes in a Beckman Ultracentrifuge L5-40 (40 rotor at 40,000 rpm). At the end of the centrifugation, the supernatant was decanted very carefully and treated with nucleases. The sample was then subjected to the daunorubicin affinity gel isolation procedures. The 100,000 g pellet was resuspended in 0.01 M KH_2PO_4 buffer at pH 7.0 and rehomogenized for 50 passes on ice (approximately 3 minutes) followed also by the nucleases treatment and the affinity gel isolation procedures.

Figure 18 demonstrates the results of the pH elution of the daunorubicin affinity gel after the gel has been incubated with the nucleases treated 100,000 g supernatant (Figure 18A) and pellet (Figure 18B) fractions. Materials with an absorption peak at approximately 275 nm and strong absorption in the 230 nm region were eluted at pH 9-9.5 from the affinity gel incubated with the 100,000 g supernatant fraction (Figure 18A). The results from the pH elution of the gel incubated with the pellet fraction showed an entirely different picture. In this case, most of the materials were eluted at pH 9.0 and these materials have a spectrophotometric absorption peak at approximately 260 nm (Figure 18B). To determine if the pH 9.5 eluate of the gel incubated with the supernatant fraction did contain the

FIGURE 18A

pH Elution Profile of the 100000g Supernatant Fraction

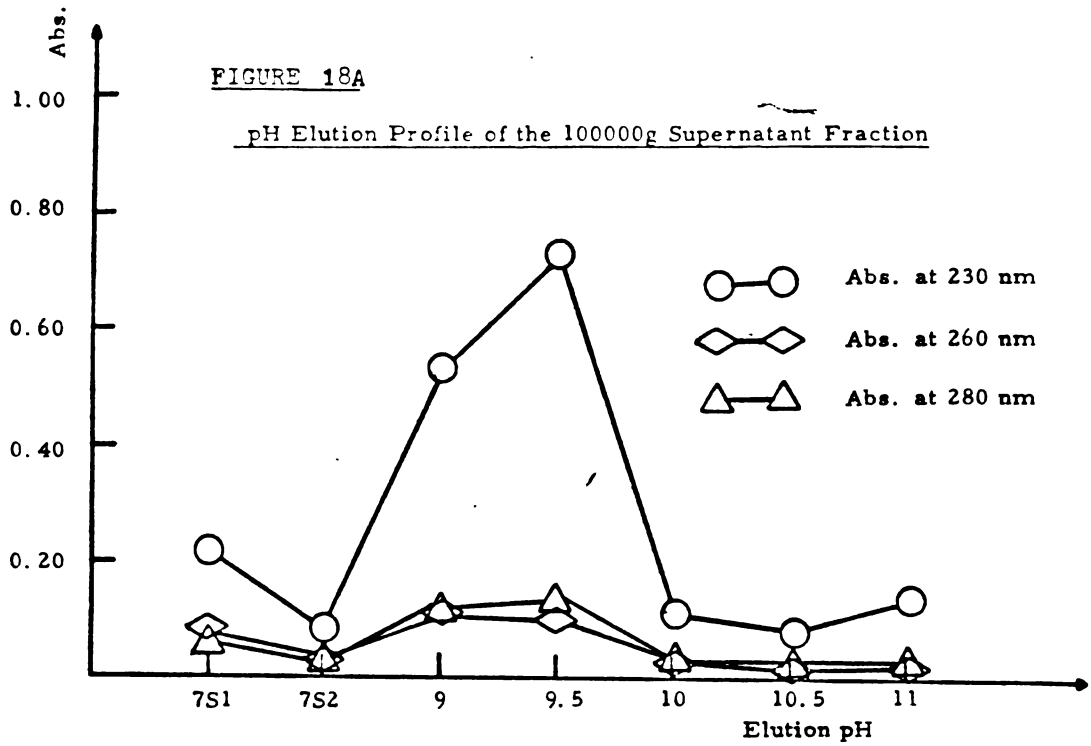
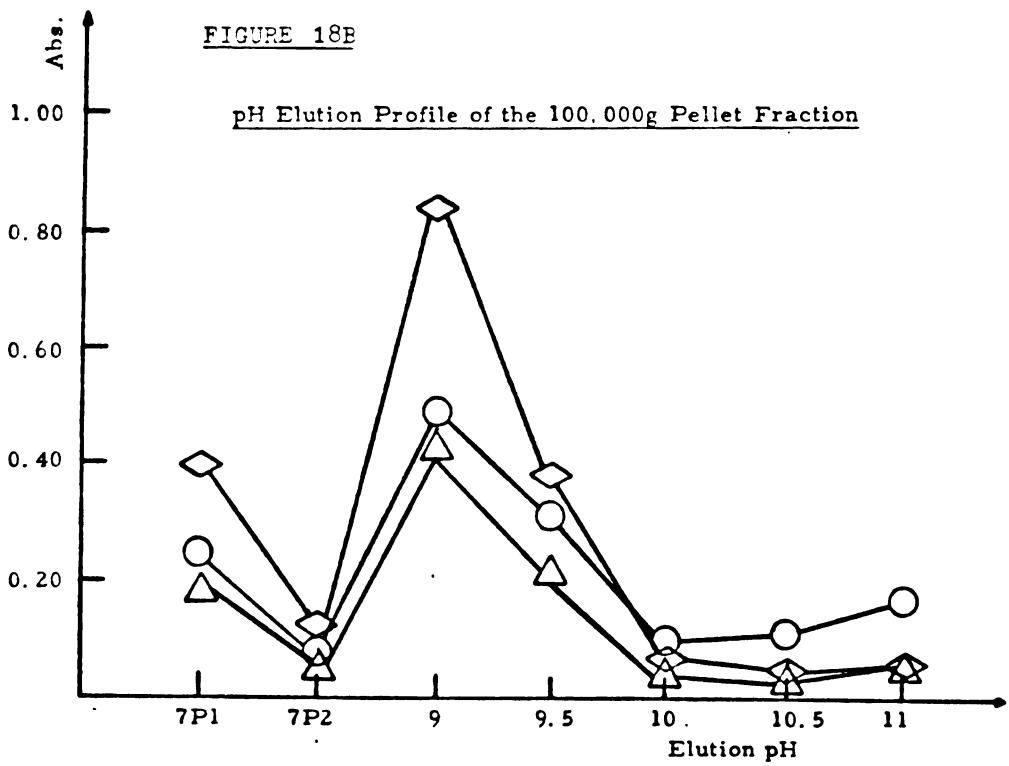


FIGURE 18B

pH Elution Profile of the 100,000g Pellet Fraction



140,000 daltons daunorubicin receptive material, the eluate was applied to the Sephadex G-200 column for analysis. Figure 19 shows the results of this experiment. The majority of the material was eluted in two peaks: a high molecular weight component with identical elution properties as the 140,000 daltons daunorubicin receptive protein described previously and a low molecular weight substance eluted in the bed volume of the column. The latter was identified spectrophotometrically as the material derived from the affinity gel and not from the heart homogenate (see p. 66 and peak III in Figure 15).

This experiment shows that the daunorubicin protein receptive material isolated from the total homogenate probably resides mostly in the soluble cytosol of the cardiac cells (although the possibility that it was released into the 100,000 g supernatant by the homogenization process cannot be ruled out) while the low molecular weight, 260 nm absorbing, material is primarily in the 100,000 g pellet fraction. The exact intracellular localization of this material is not known; if it is indeed the degradative products of the nuclease treatment (see p. 60 and p. 64), it might have come from the nuclei in the 100,000 g pellet.

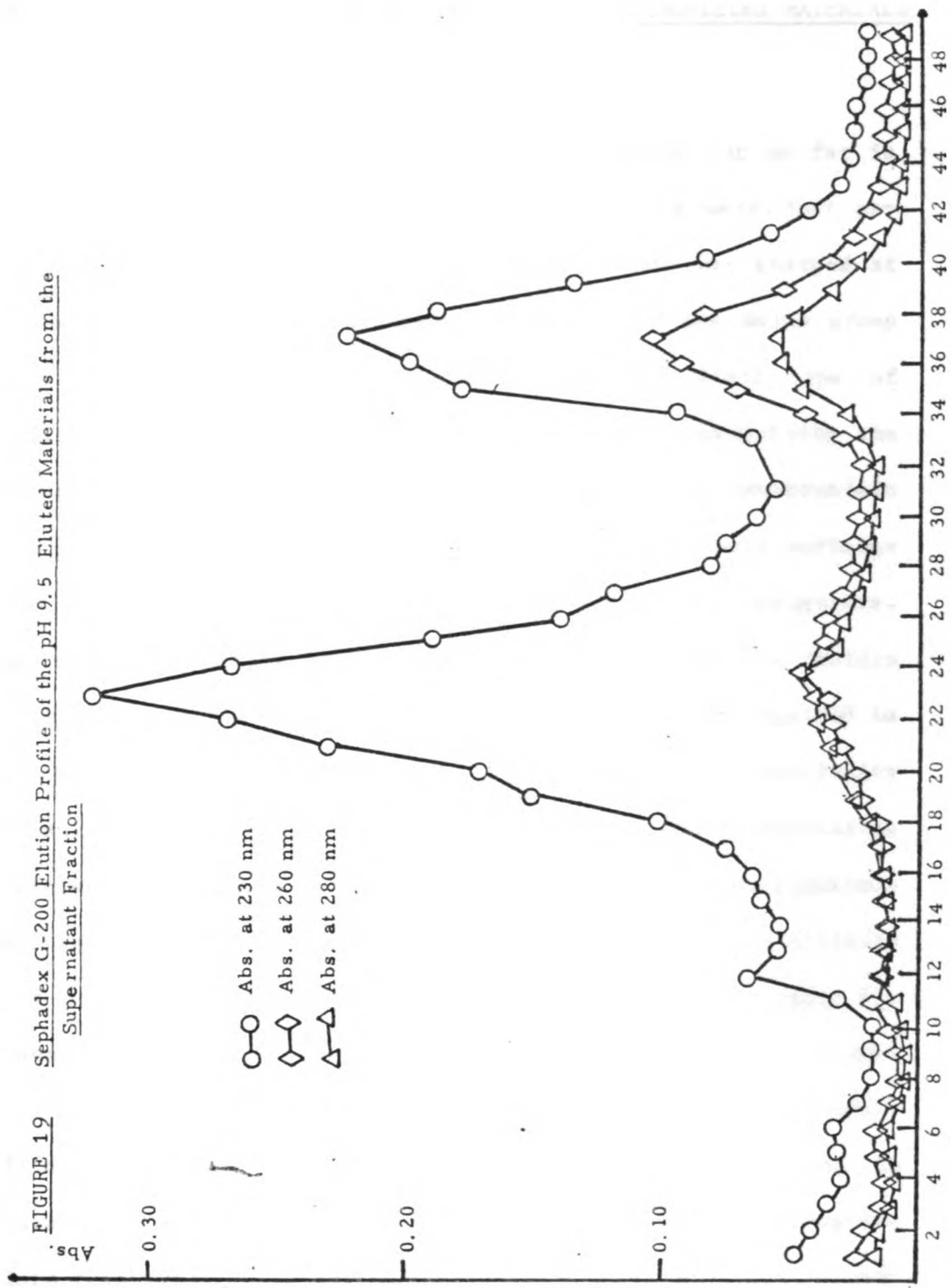
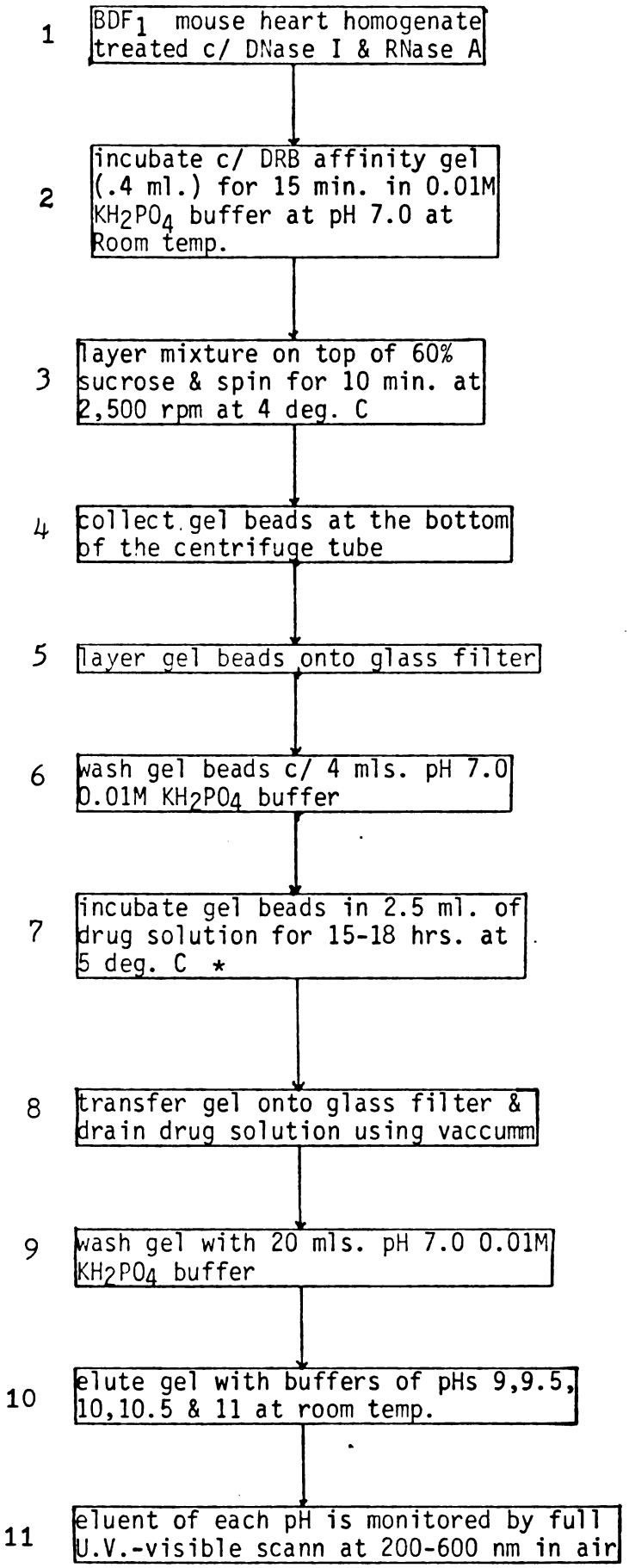


FIGURE 19 Sephadex G-200 Elution Profile of the pH 9.5 Eluted Materials from the Supernatant Fraction

L) COMPETITIVE DISPLACEMENT OF THE DAUNORUBICIN RECOGNIZING MATERIALS FROM THE AFFINITY GEL

An important possibility that has not been ruled out so far is whether the isolated daunorubicin receptive materials were just associating with the amino group of daunosamine, which was charged at the pH used in the isolation procedures (the pK_a of the amino group of daunorubicin is 8.25 (195)), in a non-specific ionic type of interaction, or were these cardiac materials really recognizing the daunorubicin molecule specifically. In other words, the daunorubicin affinity gel could just be acting as a non-specific ionic exchange resin to pull any anionic material from the total homogenate. Although this is unlikely in view of the fact that only one protein out of the many in the homogenate was pulled out, it was decided to test the hypothesis experimentally and attempt to deny the specificity of the isolated cardiac receptive substance. A series of experiments was performed to investigate the ability of different amino compounds to displace the receptive materials bound to the daunorubicin affinity gel. An outline of the procedures used is presented on Figure 20. The receptive substances from the heart homogenate were first allowed to bind to the daunorubicin affinity gel as described previously. The affinity gel with the materials bound was then incubated in solutions of various amino compounds. The percentage of the receptive materials remained bound to the affinity gel at the end of the incubation period was quantitated and compared with that of a control sample where the affinity gel had been incubated in 0.01 M KH_2PO_4 at pH 7.0 for the same period of time.

FIGURE 20



THE DISPLACEMENT OF RECEPTIVE MATERIALS FROM DRB GEL

As a positive control for the methodology, attempts were made to displace the receptive materials from the daunorubicin affinity gel, in step 7 of Figure 20, with the ligand molecule daunorubicin itself. Figure 21A is the UV-visible absorption spectrum of the pH 9.0 eluate of the daunorubicin affinity gel obtained after the affinity gel receptive substances complex had been incubated in pH 7.0 phosphate buffer for 15 hours at 4° C. As before, materials with an absorption peak at approximately 260 nm were obtained. Figure 22A represents the absorption spectrum of the pH 9.5 eluate of the same affinity gel sample with strong absorption in the 200-240 nm region. If the affinity gel-receptive substances complex was incubated in a 53 mM daunorubicin solution (0.01 M KH_2PO_4 pH 7.0) instead of the plain phosphate buffer prior to the pH elution procedures, very little material could be eluted subsequently at pHs 9.0 or 9.5 as indicated by the absorption spectra of the pHs 9.0 and 9.5 eluates (Figures 21 B and 22B).

The ability of other amino compounds to displace these cardiac receptive materials from daunorubicin affinity gel in step 7 (Figure 20) was then tested. The amino compounds tested included glucosamine, norepinephrine and histamine. Glucosamine was chosen because it was structurally similar to daunosamine, the amino sugar of daunorubicin. Previous experiments showed that the receptive materials did not recognize the daunorubicin chromophore aglycone, daunomycinone, in our experimental conditions. It is of interest to find out whether the receptive materials have an equal affinity towards the amino sugar, daunosamine, thus denying the specificity of the receptive materials to the complete drug molecule. Ideally, the amino sugar

FIGURE 21A

Displacement of Receptive Materials from DRB Affinity Gel with Daunorubicin : pH 9 Eluted Materials. Control Sample

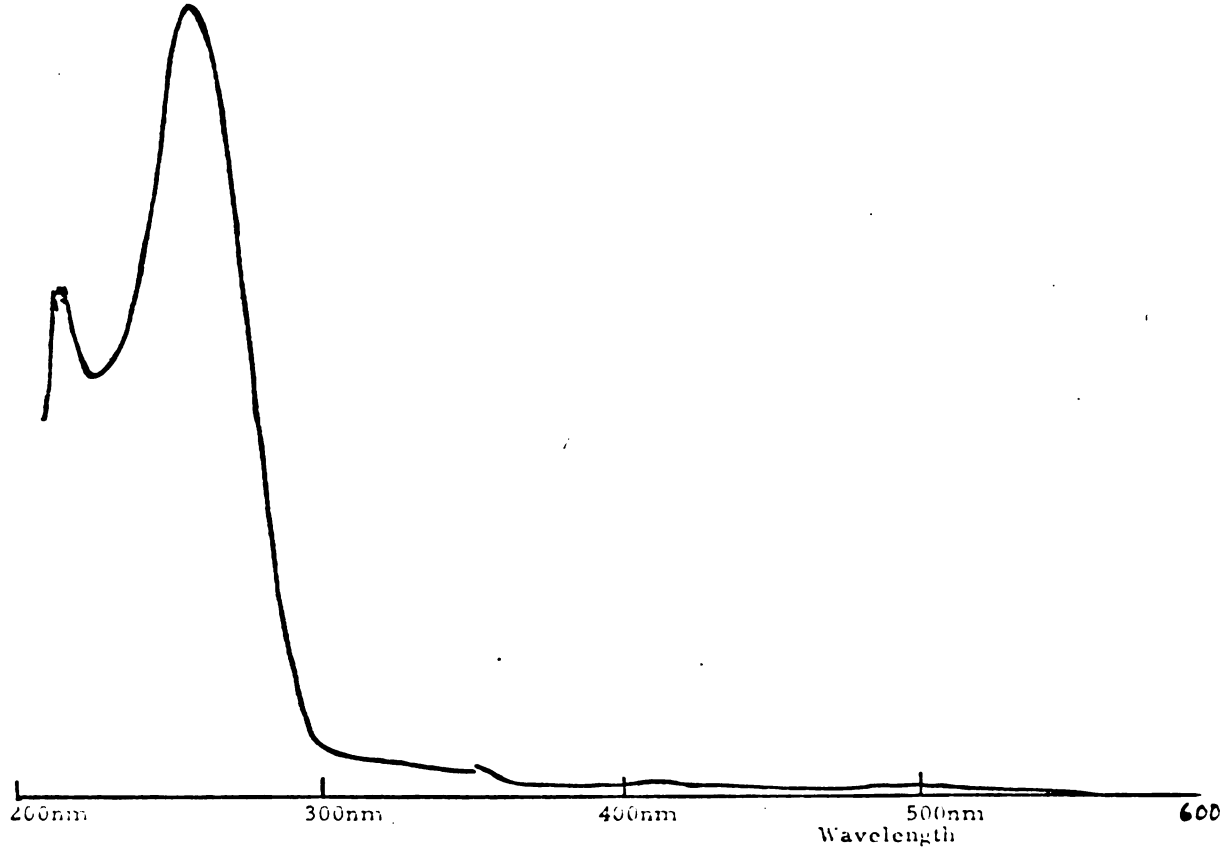


FIGURE 21B

Displacement of Receptive Materials from DRB Affinity Gel with Daunorubicin : pH 9 Eluted Materials. Exptal. Sample

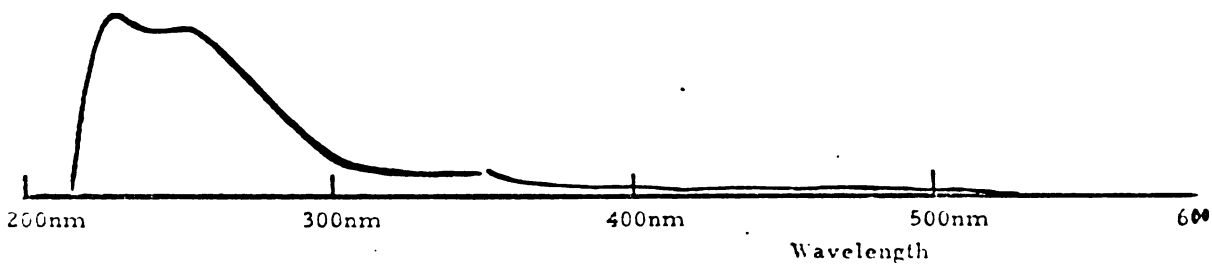


FIGURE 22A

Displacement of Receptive Materials From DRB Affinity Gel with Daunorubicin : pH 9.5 Eluted Materials. Control Sample

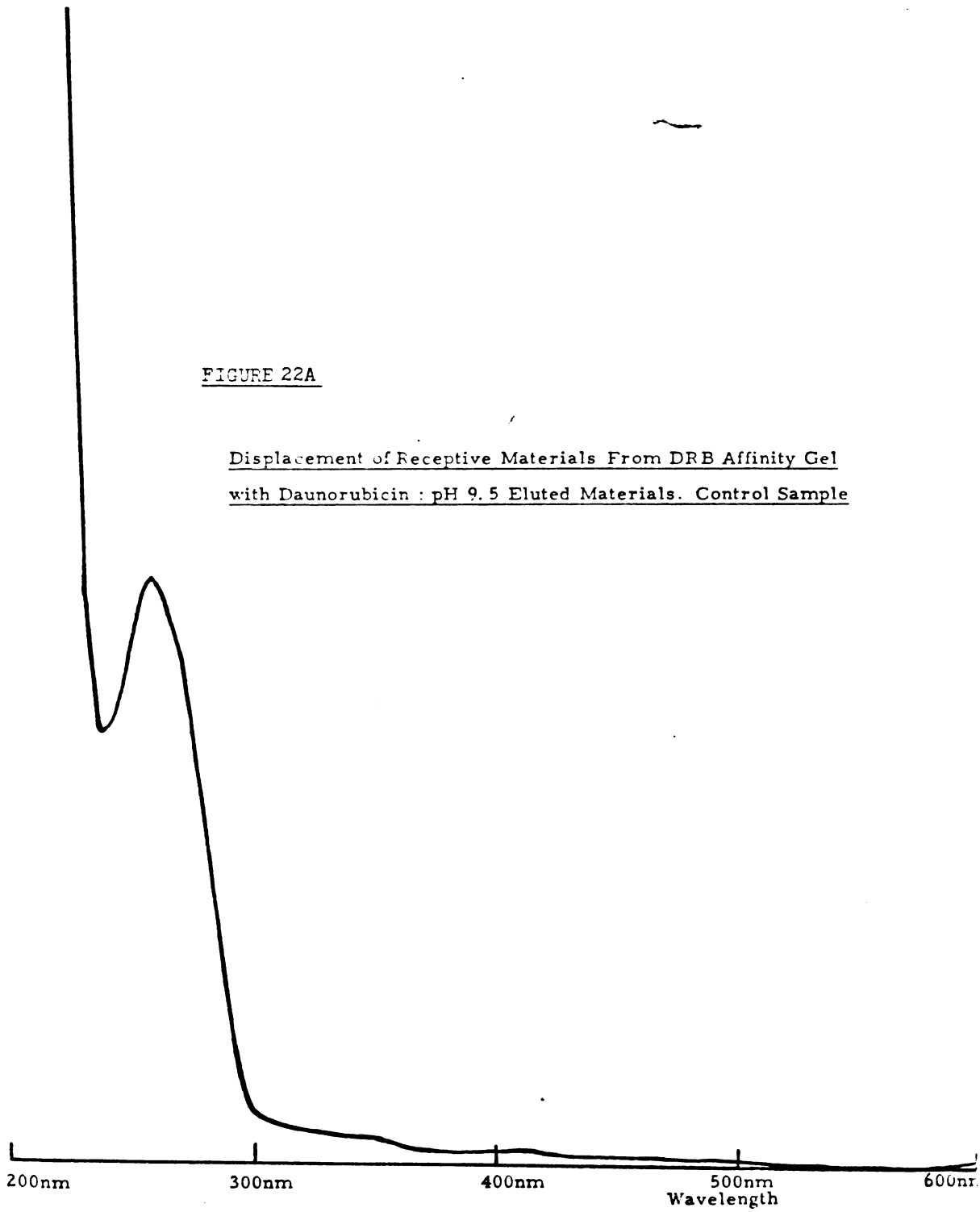
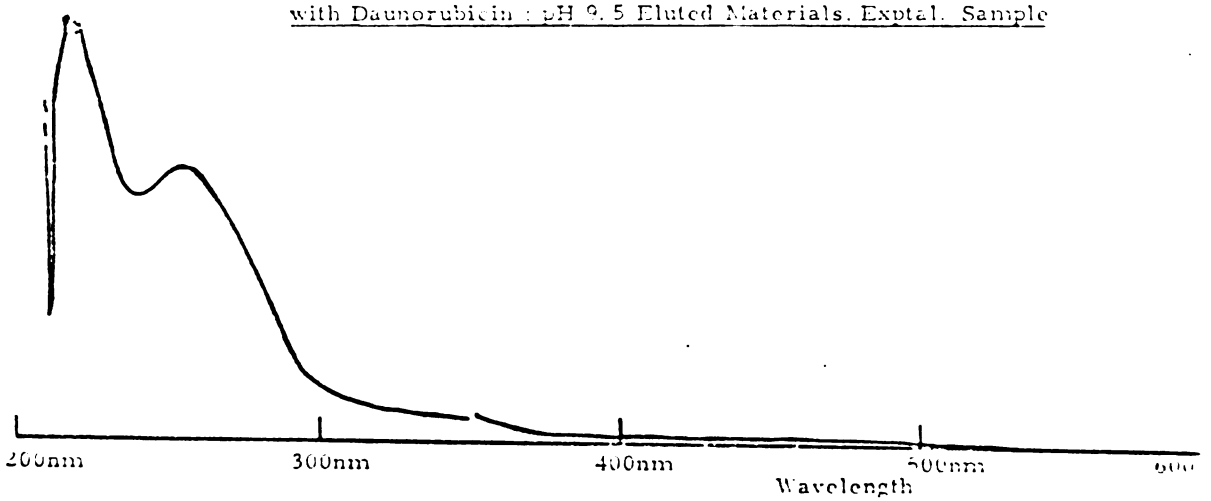
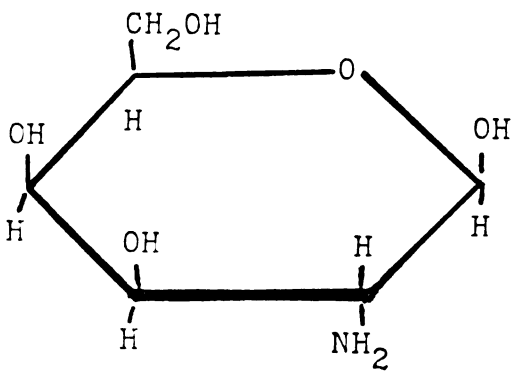


FIGURE 22B

Displacement of Receptive Materials From DRB Affinity Gel with Daunorubicin : pH 9.5 Eluted Materials. Exptal. Sample



daunosamine should be used in the experiment. Unfortunately, an adequate quantitity of this sugar was not available. Consequently, the most similar hexose commercially available, glucosamine, was used in the experiment (Sigma Chemical Company, D (+)glucosamine HCl, Catalog #G4875).



2-amino-2-deoxy-D-glucose

(glucosamine)

Like daunosamine, the amino group of glucosamine (pK_a 7.6-7.8 (196)) is expected to be largely protonated at the pH condition of our displacement experiment and if it recognizes the binding sites of the cardiac receptive materials, it should be able to displace these materials from the affinity gel. Norepinephrine (Sigma Chemical Company, DL-Arterenol catalog #A-7256) was examined because it also possessed a free amino group and the pK_a of this group was close to but slightly higher than that for daunorubicin (pK_a for norepinephrine = 8.5 (197)). If charge was the only important parameter in the observed affinity gel-receptor binding, under identical pH and molar concentration conditions, norepinephrine in solution should be more

effective in displacing the receptive materials from the affinity gel in comparison with daunorubicin. L-norepinephrine, the active isomer *in vivo*, was also tested to see if one could detect any difference between the biologically active compound and the racemic mixture in their ability to displace the receptive materials from the affinity gel. This was an approach taken to determine if the isolated daunorubicin receptive materials, especially the protein substance, was identical to the norepinephrine receptor in cardiac cells. Norepinephrine is known to cause cardiac arrhythmia (198) and it is possible that the arrhythmic property of the anthracyclines is due to their stimulation of the norepinephrine receptors on heart cells. If this is indeed the case, one should be able to get a positive displacement result in the experiment and the L-norepinephrine should be more effective than an equal concentration of the racemic mixture.

Histamine reactions such as flushing of the skin and local and systemic reactions including urticaria, erythema and analphylaxis have been observed in patients after anthracycline administration (199-202) and some of these symptoms could be prevented or treated with antihistamine medications (200). Recently, Herman demonstrated that adriamycin-induced hypotension in the Beagle dog could be suppressed by tripelennamine, an antihistamine (203). These observations led to the hypothesis that the anthracyclines might be exerting some of their effects by stimulating endogenous histamine receptors. Consequently, histamine (Sigma Chemical Company, histamine dihydrochloride catalog #H7250) was included in the cardiac receptive materials displacement experiments to find out if these materials had any affinity towards

histamine.

Table 3 summarizes the results of these experiments. Daunorubicin, at a concentration of 0.2 M, was not very effective in dislodging the cardiac materials from the daunorubicin affinity gel. As the drug concentration increased, a higher and higher percentage of the receptive substances was displaced. In general, the pH 9.5 eluted materials containing the 140,000 daltons receptive protein was less readily displaced than the pH 9.0 eluted materials, consisting mainly of the low molecular weight 260 nm absorbing species. With a fifteen hour incubation at 4° C in a 53 mM daunorubicin solution, as much as 85% of the pH 9.0 eluted materials and 65% of the pH 9.5 substances were displaced. Higher concentrations of daunorubicin were not tested because of the large amount of drug required. In contrast, none of the other amino compounds tested was very effective. Since all the chemicals used in these experiments were in the hydrochloride form (histamine 2 HCl, norepinephrine HCl and glucosamine HCl), the effect of sodium chloride in the displacement experiment was investigated as the control for the salt effects. Results showed that although NaCl (60 mM) was capable of dissociating some of the pH 9.0 eluted materials from the affinity gel, it had no effect on the binding of the pH 9.5 eluted substances. Therefore, the 20-30% "displacement" of the pH 9.0 eluted materials by histamine, norepinephrine and glucosamine observed was probably due to a salt effect resulting from the addition of the drugs as their hydrochloric salts, rather than a specific displacement by the compounds.

TABLE 3 THE DISPLACEMENT OF DAUNORUBICIN RECEPTIVE MATERIALS BY AMINO COMPOUNDS

Drug	Concentration	Incubation Conditions	% displacement at 260 nm	
			pH 9 Eluent	pH 9.5 Eluent
Daunorubicin	0.2mM	8 mins. @R.T.	0.7%	-8%
Daunorubicin	18 mM	1 hr. @ R.T.	37%	5%
Daunorubicin	35 mM	4 hrs. @ R.T.	60%	18%
Daunorubicin	35 mM	18 hrs. @ 4°C	70%	40%
Daunorubicin	53 mM	15 hrs. @ 4°C	85%	65%
Histamine	35 mM	18 hrs. @ 4°C	34%	4%
Nor-Epi(d+l)	35 mM	18 hrs. @ 4°C	20%	1%
l-NorEpi	53 mM	15 hrs. @ 4°C	20%	-5%
Glucosamine	53 mM	15 hrs. @ 4°C	30%	5%
NaCl	60 mM	18 hrs. @4°C	25%	-3%

Previous experiments showed that the chromophore of daunorubicin alone was not sufficient for the cardiac receptive materials binding. Together with the results obtained in the displacement experiments, it appears that the complete molecule of daunorubicin is necessary for the binding to take place. Unfortunately, daunosamine was not available for testing for a more definitive answer.

M) THE ISOLATION OF CARDIAC COMPONENTS WITH ADRIAMYCIN AFFINITY GEL

A protein material from the mouse heart that recognizes daunorubicin and appears to have a very high specific affinity for the drug has been identified. But does the recognition of this material extend to other structurally similar anthracycline antibiotics or is it specifically a daunorubicin "receptor"? To approach this problem, another readily available anthracycline antibiotic presently used very extensively for cancer treatment, adriamycin, was chosen to be examined.

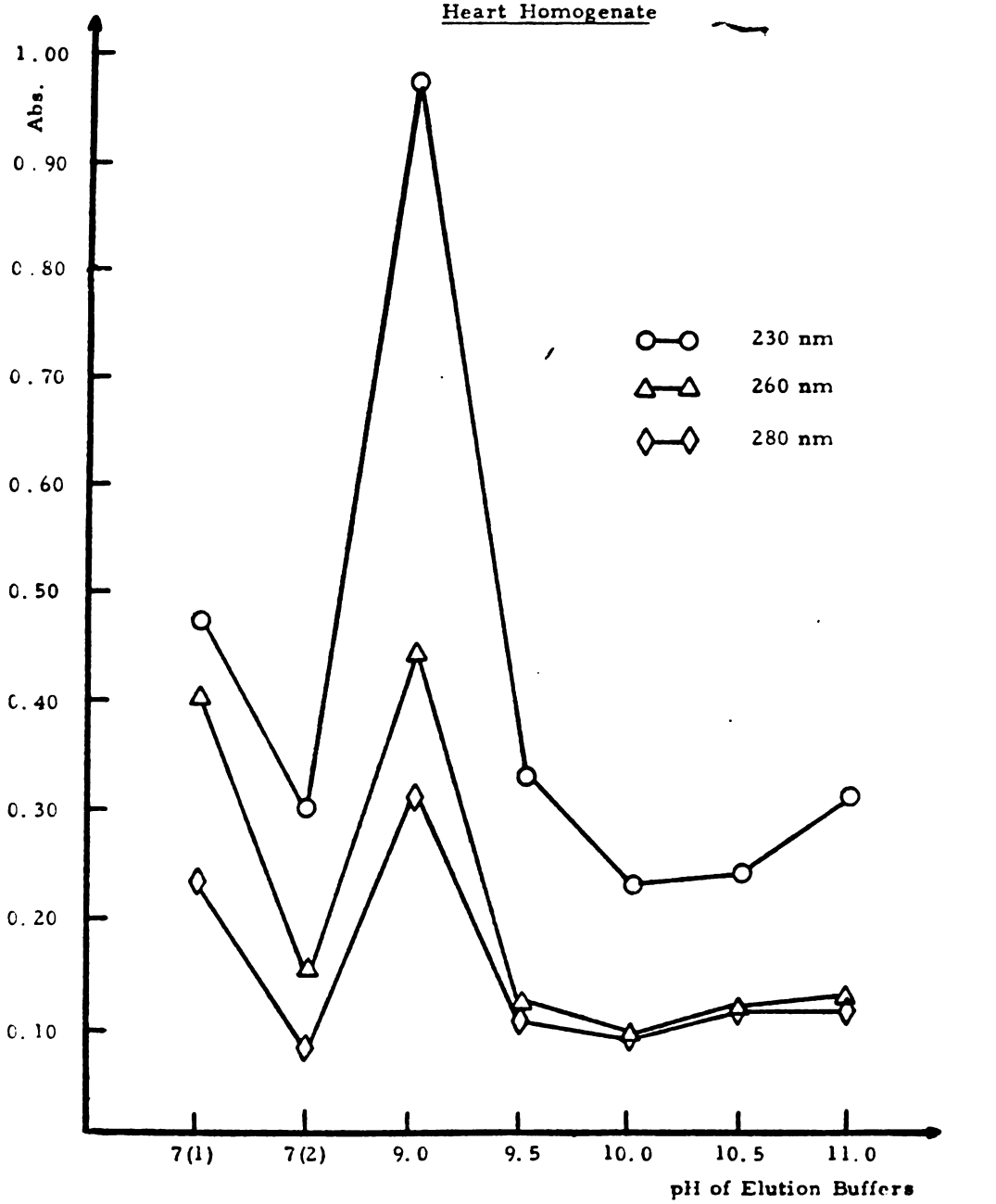
The question we are asking is, "Will the daunorubicin receptive materials recognize adriamycin too?" To answer this question, the receptive materials displacement procedure was employed. The ability of adriamycin to displace the cardiac receptive substances from the daunorubicin affinity gel was investigated. By looking at the ability of adriamycin to displace in comparison with daunorubicin, one might be able to get an estimation of the relative affinity of these two anthracyclines towards the receptive materials. To carry out the experiment, the cardiac substances were first bound to the daunorubicin affinity gel in the usual manner and the complex was then incubated in an adriamycin solution. At the end of the incubation, the percentage of the receptive materials remaining on the gel was quantitated. Although 35 mM of adriamycin was able to displace all the receptive substances from the daunorubicin affinity gel after an incubation period of 18 hours at 4^{o} C, there was a problem in the interpretation of this data. Adriamycin adsorbed to the affinity gel to a great extent and it took a prolonged period of washing with large

volumes of 0.01 M KH_2PO_4 buffer to wash off the drug sticking to the affinity gel at the end of the gel-adriamycin incubation period, before the pH elution step could be carried out. Consequently, another approach must be used.

Adriamycin ligand affinity gel was synthesized using the same method employed in the synthesis of the daunorubicin affinity gel and the behavior of this affinity gel in the cardiac receptor isolation protocol was investigated. Figure 23 depicts the pH elution profile of the adriamycin affinity gel after the gel has been incubated with nuclease-treated mouse heart homogenate. There was a slight modification of the procedures used in this experiment. After the affinity gel was sedimented in 60% sucrose and placed on glass filter, it was washed twice with 2 ml of 0.01 M KH_2PO_4 pH 7.0 buffer (a total of 4 ml) instead of washing it with 2 ml of the phosphate buffer (see Figure 7) as was done previously, to ensure a more complete washing of the gel before increasing the elution pH. On the graph of Figure 23, the data points 7(1) and 7(2) represent the first and second two ml of the pH 7.0 phosphate washes before the elution pH was raised to pH 9.0. Unlike the results obtained for the daunorubicin affinity gel (Figure 8), most of the materials were eluted off the adriamycin affinity gel at pH 9.0 while the eluates of the higher pHs showed some general absorption in the 225-240 nm region. Judging from the intensity of the color that came off the gel with elution at high pHs, adriamycin appeared to be more susceptible to dissociation from the gel matrix at the more basic pHs in comparison with daunorubicin (see p. 47). The pale violet color of the eluates of higher pHs

FIGURE 23

pH Elution Profile of Adriamycin Affinity Gel After Incubation with Mouse Heart Homogenate

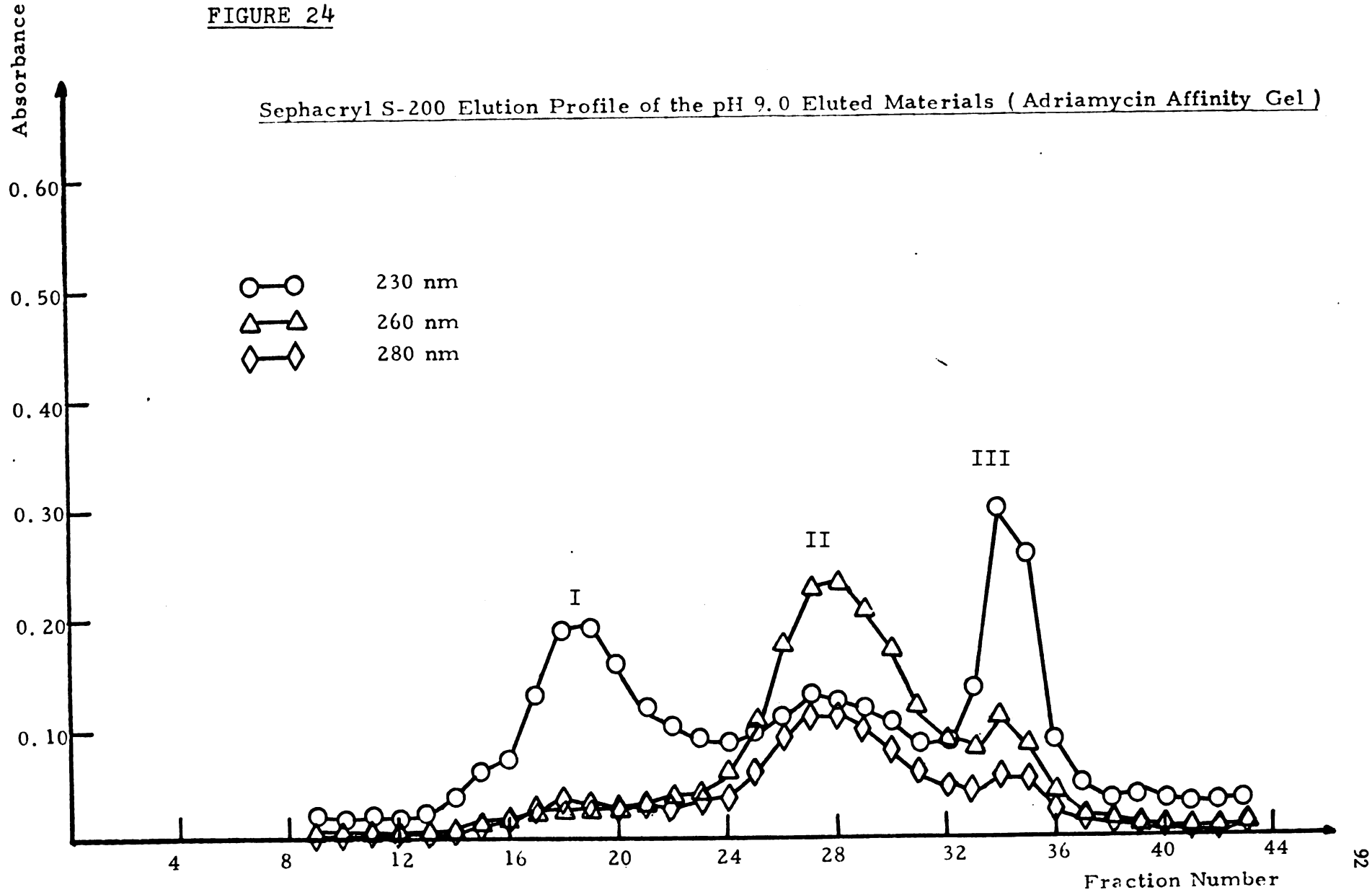


(pH 9.5-11.0) disappeared the next morning (samples were stored in the refrigerator overnight) when the eluates were analyzed spectrophotometrically, indicating that the chromophore of the drug was probably not stable at alkaline pH.

The pH 9.0 eluate from the adriamycin affinity gel was subsequently subjected to gel filtration chromatography for sizing. Previous experience with Sephadex gels was not very satisfactory, especially with the Sephadex G-200 gel system. The gel beads were so compressible that the column became clogged after running a few samples and the flow rate was extremely low. Consequently, a new gel system Sephacryl S-200 (Pharmacia Fine Chemical Sephacryl S-200 superfine) was utilized in these experiments. Sephacryl S-200 behaves similarly to Sephadex G-200 in the chromatographic elution characteristics of macromolecules and it has better rigidity and reasonably good flow properties. Figure 24 shows the elution profile of the adriamycin affinity gel pH 9.0 eluate on a Sephacryl S-200 column. It could be resolved into three components: a high molecular weight component and two components with low molecular weights. The high molecular weight material (peak I in Figure 24) had a spectrophotometric absorption peak at approximately 275 nm and strong absorption in the 200-250 nm region, and was eluted from the Sephacryl column at the same position as the daunorubicin recognizing protein isolated previously using daunorubicin affinity gel, and lactic dehydrogenase from the beef heart (M.W. 140,000). SDS-polyacrylamide gel electrophoresis of this material showed one single protein band with the same migration characteristic (as measured from a cytochrome C band

FIGURE 24

Sephacryl S-200 Elution Profile of the pH 9.0 Eluted Materials (Adriamycin Affinity Gel)



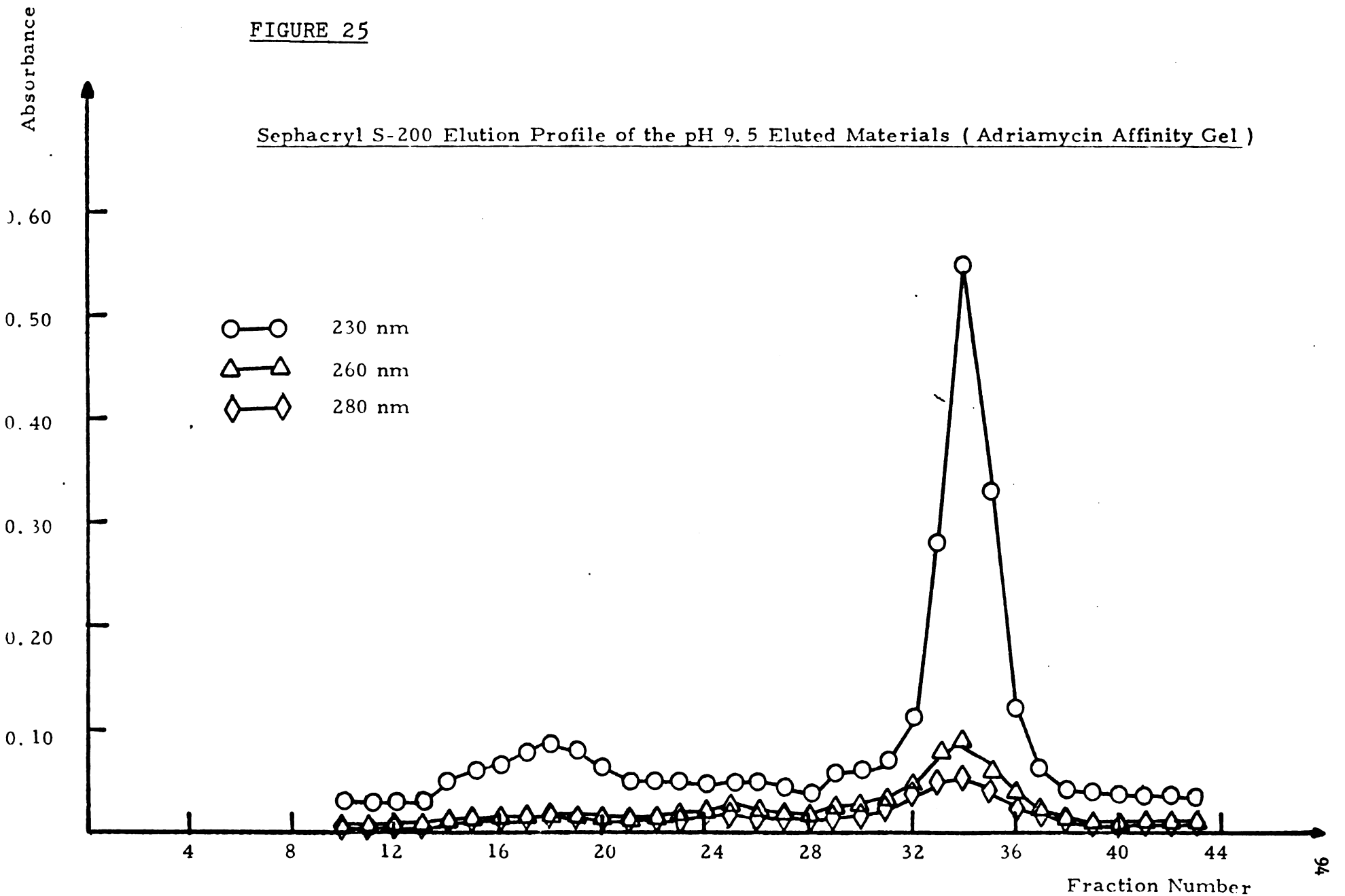
added as a reference protein band) as the low molecular weight band of the daunorubicin recognizing protein (Figure 17) (molecular weight approximately 65,000 daltons). Unexpectedly, after Sephacryl gels the high molecular weight protein band with 140,000 molecular weight seen in the daunorubicin receptive protein's electrophoretic run (Figure 17) was not observed in this experiment for either the pH 9.5 eluate from the daunorubicin affinity gel or the pH 9.0 eluate from the adriamycin affinity gel. In spite of using the same electrophoretic conditions, repeated experiments with pH 9.5 daunorubicin affinity gel eluates afterwards never confirmed previous SDS-electrophoretic results where both the M.W. 140,000 and the 65,000 daltons protein bands were observed, although the latter component appeared consistently in all the experiments. The reason for this discrepancy is unknown.

Materials in peak II of the Sephacryl S-200 elution profile (Figure 24) had an absorption peak at approximately 260 nm and resembled spectrophotometrically the component in the daunorubicin gel eluates which was believed to be the degradative products of the nuclease treatment (peak II of Figure 15). A final component (peak III in Figure 24) exhibited identical spectrophotometric characteristics to the material eluted from the daunorubicin affinity gel shown to have derived from the gel and not from the heart homogenate (peak III in Figure 15 and see discussion on p. 64-66).

The pH 9.5 eluate of the adriamycin affinity gel was also analyzed with the Sephacryl S-200 column and its elution profile is shown in Figure 25. Most of the materials in this eluate were composed of substances with identical chromatographic and spectrophotometric

FIGURE 25

Sephacryl S-200 Elution Profile of the pH 9.5 Eluted Materials (Adriamycin Affinity Gel)



properties as the material in peak III of the Sephacryl separated pH 9.0 adriamycin affinity gel eluate (Figure 24).

From the results of these experiments, it appears that the daunorubicin receptive materials do also recognize adriamycin. Higher quantities of the cardiac substances could be isolated using daunorubicin affinity gel than could be accomplished with an equal volume of the adriamycin gel. This seems reasonable because the daunorubicin gel has a higher ligand capacity than its adriamycin counterpart (see p. 39). The receptive protein which was eluted off the daunorubicin affinity gel at pH 9.5 dissociated from the adriamycin ligand gel at pH 9.0. This might be due to a difference in the pK_a of the amino group in the two compounds. Skovsgaard reported a pK_a of 8.25 for daunorubicin and 8.15 for adriamycin (195). This results in a lesser degree of ionization for adriamycin at the pH of the pH 9.0 elution and may explain why the receptive protein dissociated from adriamycin affinity gel at a lower pH in comparison with daunorubicin.

N) ISOLATION OF MEMBRANE ASSOCIATED DAUNORUBICIN RECOGNIZING MATERIALS

Since our receptor isolation procedures involve the sedimentation of the affinity gel through 60% sucrose solution at 4° C, a question is raised whether the "receptor-drug" coupling is strong enough to pull membrane associated receptive materials down through the dense sucrose solution and be subsequently isolated. The anthracyclines have been reported to alter the surface properties of cultured cells, inhibit sodium-potassium-activated adenosine triphosphatase from heart microsome and interfere with mitochondrial energy production (see p. 16-18), all of which implicate the possible interaction of the anthracyclines with membrane components. Consequently, a study was initiated to find out if one can isolate additional receptive materials from the mouse heart homogenate whose membrane components have been solubilized by detergent treatment.

Mouse heart homogenate was prepared as previously described (p. 46) and centrifuged at 100,000 g for 60 minutes. The supernatant which contained the 140,000 daltons receptive protein was decanted carefully. The pellet fraction was resuspended in four ml of 0.01 M KH_2PO_4 buffer at pH 7.0 and rehomogenized for 60 passes for approximately three minutes. The suspension was then separated into two portions of equal volume. 0.05 ml of triton x-100 (Sigma Chemical Company) was added carefully to one of the portions (portion A) while an equal volume of the 0.01 M phosphate buffer was added to the other (portion B). The two samples were then gently shaken at room temperature for a period of ninety minutes. At the end of the incubation

period, the samples were dialyzed against 0.01 M KH_2PO_4 at pH 7.0 at 5° C for twenty-four hours. The samples were then subjected to the daunorubicin affinity gel isolation procedures (Figure 7).

Triton x-100 is an alkylphenylpolyoxyethylene condensate (average M.W. 628) which has been used extensively for the solubilization of membrane structures in the fields of pharmacology, cell and molecular biology and biochemistry (204). It solubilizes membrane very efficiently and, because of its non-ionic character, it can be included in situations where the ionic environment is a critical consideration such as in the cases of ion exchange chromatography and some receptor-ligand binding experiments (205-206). Although non-ionic detergents in general are not as effective in their ability to solubilize membrane in comparison with the ionic type of detergents, they are usually more gentle in their denaturation action and cause far less conformational changes in membrane components than their ionic counterparts. The degree of membrane solubilization depends on the detergent concentration. With 1% (v/v) triton x-100, Cuatrecasas was able to release 80% of the ^{125}I-labelled insulin binding activity of fat or liver membranes into the high-speed centrifugation (300,000 g x 2 hours) supernatant (205). On the other hand, 1.5% triton x-100 (v/v) was required to bring 80% of the tritiated-burgarotoxin binding materials in the tougher rat diaphragm muscles into solution (207). In our experiment, a 2.0% triton x-100 solution was used to ensure more complete membrane solubilization. With this concentration of the triton x-100, the turbidity of the resuspended 100,000 g pellet fraction was markedly reduced and assumed a relatively clear pinkish color. In the

experiment, the triton x-100 was not removed before the fraction was subjected to the daunorubicin affinity gel isolation procedures. The detergent has the tendency to form micelles in aqueous solution and behaves as a large molecular weight aggregate (208-209). Hence, the dialysis step was not effective in removing the detergent from the homogenate. However, it was felt that since any influence that the detergent might have on the binding of cardiac components with the daunorubicin affinity gel would be hydrophobic in nature and our isolation procedures would not pick up materials weakly hydrophobically bound to the gel anyway because of the 60% sucrose centrifugation step (see p. 45), it was unnecessary to remove the detergent. This notion was supported by the observation that the isolation of the 140,000 daltons daunorubicin recognizing protein from the 100,000 g supernatant was not affected by the presence of triton x-100 in the same concentration. Furthermore, there has been experimental evidence suggesting that the membrane components might reaggregate upon the removal of the detergent (209-212). Therefore, it was decided to run the isolation procedures without removing the triton x-100. Some detergent stayed adhered to the affinity gel after the centrifugation step in 60% sucrose (see Figure 7) and it was necessary to wash the gel with 6 ml of 0.01 M KH_2PO_4 buffer at pH 7.0 to clear the gel of the detergent before the pH elution could be carried out. Materials with an absorption peak at approximately 260 nm and very little absorption in the 200-240 nm region were obtained when the elution pH was raised to 9.0. At pH 9.5, substances with broad absorption in the 200-275 nm region were eluted. These materials were subjected

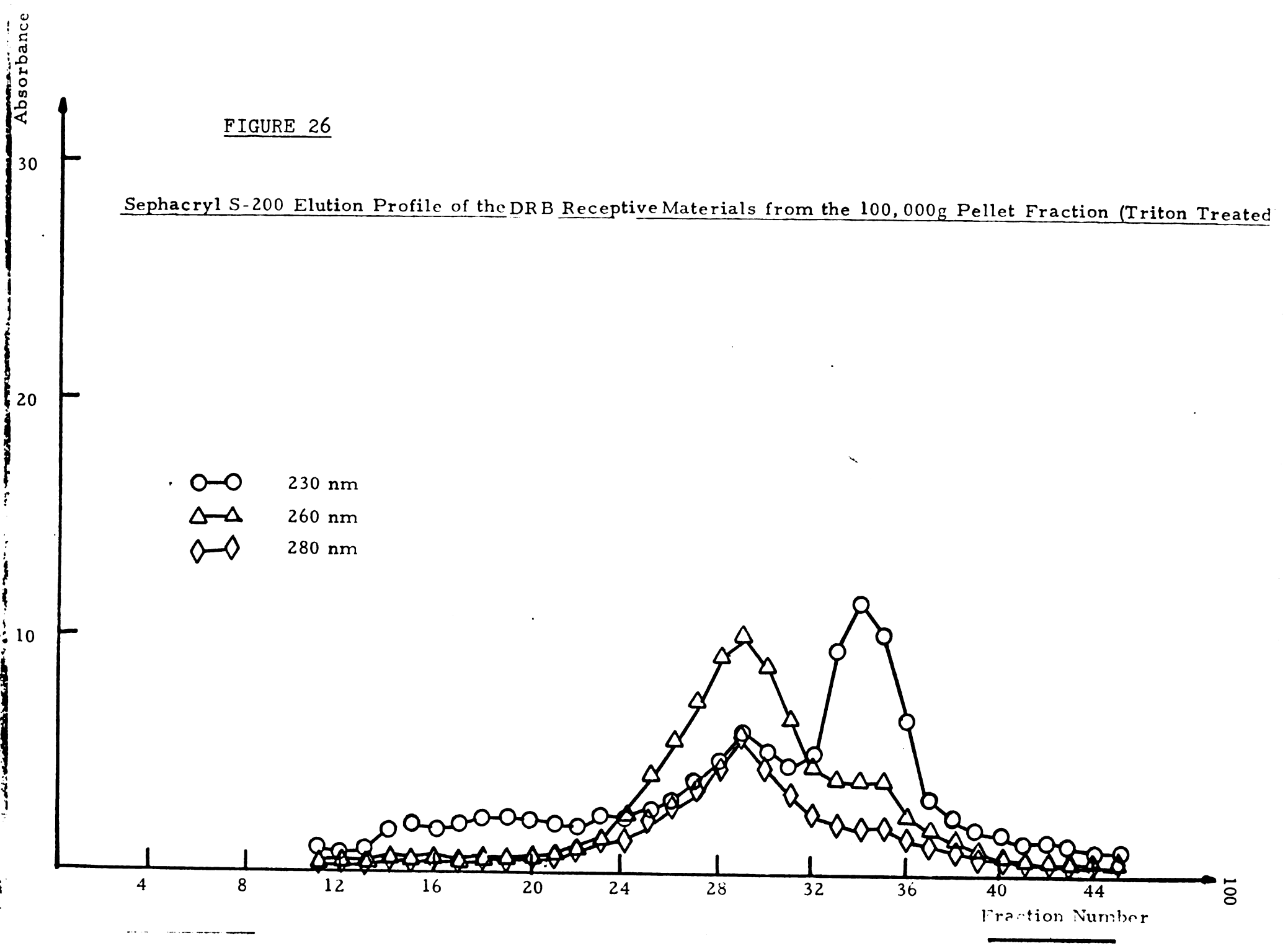
to Sephacryl S-200 chromatography and the result is shown in Figure 26. Since this was the same Sephacryl S-200 column used in the analysis of materials eluted from the adriamycin affinity gel after the gel had been incubated with the nucleases treated heart homogenate (Figure 24), one can compare the elution profiles of the two experiments. Only two components were observed in Figure 26 which corresponded both chromatographically and spectrophotometrically to peak II and peak III on Figure 24 (see p. 93 for the identification of the materials in these peaks); no material could be detected in the high molecular weight region of the Sephacryl elution profile. So it appeared that the treatment of the resuspended 100,000 g pellet fraction with triton x-100 did not result in the solubilization of any membrane components that would recognize the daunorubicin affinity gel under our experimental conditions.

In the course of the experiment, it was noticed that the quantity of receptive materials isolated from the triton x-100 treated 100,000 g pellet fraction was much lower than that which could be obtained from a pellet fraction untreated with the detergent under the same conditions. Apparently, the presence of triton x-100 in the homogenate in some way reduced the amount of materials that could be bound to the affinity gel. Consequently, efforts were made to remove the detergent from the homogenate prior to its incubation with the affinity gel. This turned out not to be a trivial problem.

The difficulty in removing triton x-100 from the sample results mainly from the non-ionic nature of the triton molecule and its extremely low critical micelle concentration. The critical micelle

FIGURE 26

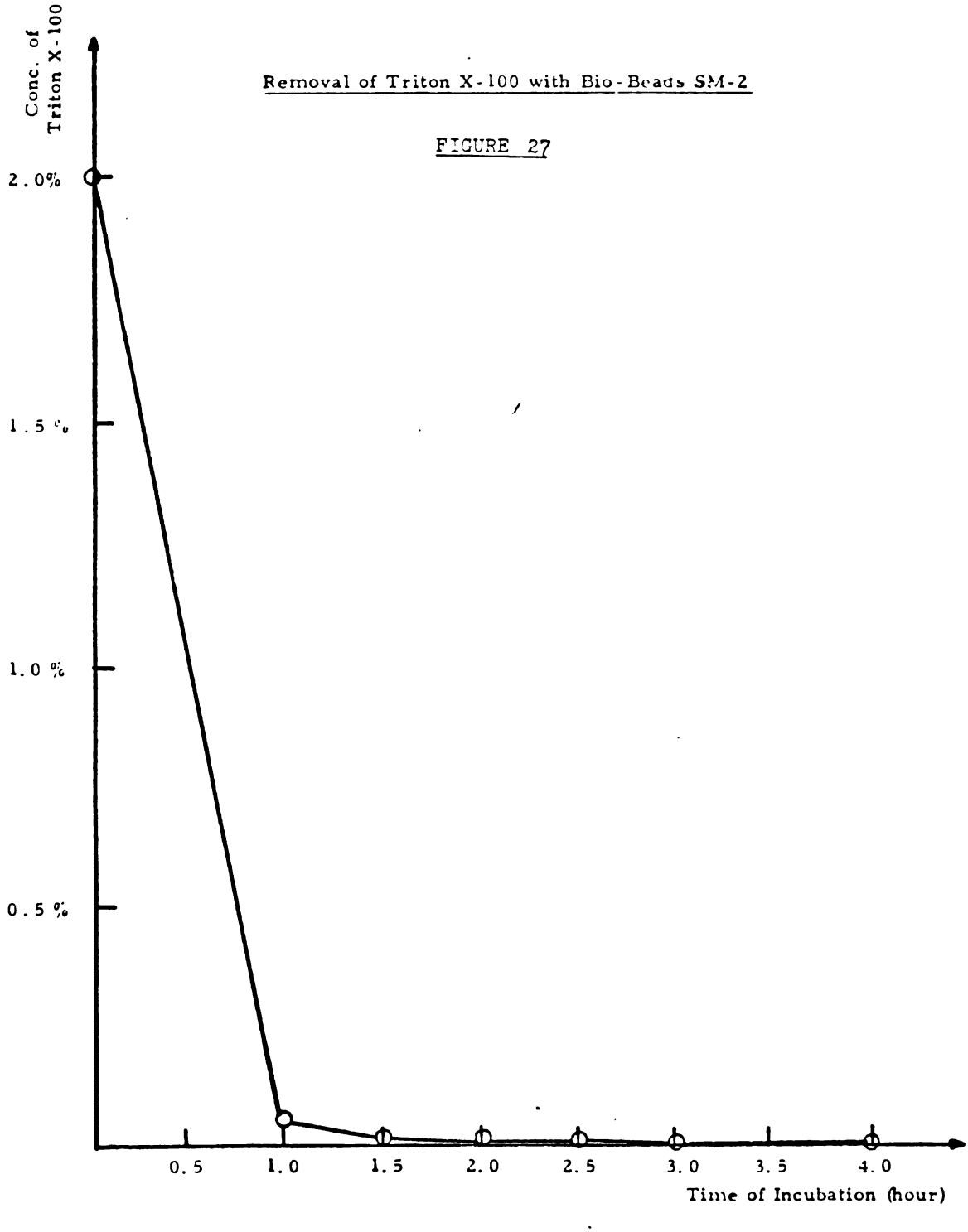
Sephacryl S-200 Elution Profile of the DRB Receptive Materials from the 100,000g Pellet Fraction (Triton Treated



concentration of triton x-100 was reported to be 0.017% at 37° C in 60 mM potassium phosphate at pH 7.2 (213) or 0.01% under unspecified conditions (214). As a result, the detergent behaves as a large molecular weight aggregate in aqueous environments in spite of its low intrinsic molecular weight. Any attempt to remove it as low molecular weight species using techniques such as dialysis or gel filtration is destined to fail. One can perhaps take advantage of the low density of these detergent micelles and utilize sucrose density gradient centrifugation for its removal. But this technique is very time consuming and is therefore inappropriate for our purpose, as this would greatly increase the probability of denaturation of our cardiac components. After numerous frustrated attempts, it was finally discovered that triton x-100 has a very high affinity towards a neutral porous styrene-divinylbenzene copolymer bead commercially available (BioRad Laboratories, Bio-Beads SM-2 catalog #152-3920). This copolymer is in the form of rigid, insoluble tiny gel beads (molecular exclusion limit 14,000) which can be removed from the sample by simple filtration. Figure 27 illustrates the effectiveness of this material to remove triton x-100 from 0.01 M KH_2PO_4 buffer at pH 7.0. The concentration of triton x-100 remained in solution was followed spectrophotometrically at 275 nm. As much as 95% of the detergent was removed by the beads within one hour and at the end of three hours, the concentration of triton x-100 in solution was reduced to 0.006% which was already below the critical micelle concentration. The concentration of protein present in the solution was reported not to be affected significantly by this treatment of the SM-2 beads (215).

Removal of Triton X-100 with Bio-Beads SM-2

FIGURE 27



Using this procedure followed by 20 hours of dialysis at 4° C, one can remove all detectable trace of the triton x-100 from the phosphate buffer. This methodology was then applied to the triton treated pellet fraction before the fraction was incubatd with daunorubicin affinity gel in the receptor isolation procedures. The results showed that even after the elimination of the detergent, no new material could be isolated from the triton x-100 treated 100,000 g pellet fraction using the daunorubicin affinity gel. However, due to the many manipulations of the homogenate (triton x-100 treatment, SM-2 beads treatment, dialysis) prior to its incubation with the affinity gel, it is very possible that there might indeed be small amounts of other daunorubicin recognizing substances that were solubilized by the detergent but were, during the preparation of the sample, altered to become less specific or effectively detected.

CONCLUSIONS

Based on the results obtained from this study, we can deny the hypothesis that DNA is the only high affinity cardiac receptor for the antitumor anthracycline antibiotics. Using a newly developed affinity gel isolation procedure, a protein was isolated from the mouse heart that recognized daunorubicin and adriamycin specifically and with high affinity. The isolated protein has the following properties:

- This protein recognizes the active antitumor anthracyclines adriamycin and daunorubicin specifically but does not have an affinity towards the inactive metabolite of daunorubicin, daunomycinone.

- This protein is mainly localized in the cytoplasm of cardiac cells.

- Sephadex gel filtration chromatographic analysis indicated that this protein has an apparent molecular weight of approximately 140,000 daltons.

- Under SDS-polyacrylamide gel electrophoretic conditions, the protein can be dissociated into subunits with molecular weight of approximately 65,000 daltons.

- This protein is distinct from the histamine and the norepinephrine receptors in cardiac cells as evidenced from its lack of affinity towards histamine and norepinephrine. It also does not have any significant affinity towards glucosamine, an amino sugar structurally similar to daunosamine, the hexose attached to the aglycone chromophore of the anthracycline antibiotics.

The most important hypothesis to be tested at this point is that the interaction between this material and the anthracyclines is indeed related to the cardiotoxicity induced by these agents. Since the drug response we are dealing with here is gross cell damage, we do not have the luxury of working with a specific, immediate easily measurable physiological reaction resulting from the drug-receptor interaction as in some other fields of pharmacology (e.g. a change in the ion permeability, the ion flux or the membrane potential resulting from the binding of neurotransmitters to their receptors). Furthermore, we do not have the advantage of having agonist-antagonist pairs from which we can generate a great deal of information. It becomes exceedingly difficult to show a cause and effect relationship between the binding of some cell component with the drug molecule and the ultimate physiological response observed. What is more complicated is the possibility that there might be more than one such interaction that is contributing to the final picture of cardiac cellular damages.

Preliminary experiments showed that this anthracycline receptive protein might also be present in the mouse liver. However, this does not necessarily negate the involvement of this protein with the cardiotoxicity seen. The physiological role the heart plays in the body makes it a very special organ. Its constant performance as a pump throughout the lifetime of the organism is a basic condition for the circulation of blood and therefore for the oxygen supply to the whole body whose absence would mean instant death. This uninterrupted vigorous mechanical activity distinguishes the heart from the

other organs. Therefore, the binding of the drug with a constituent in the heart may conceivably have a very different significance compared to the binding of the same component in the liver. In addition, the liver is known for its remarkable activity in drug metabolism and the fate of an agent in the liver might very well be dissimilar than that in the heart. Anthracyclines are also hepatotoxic, but this is much more reversible in the rapidly regenerating liver tissue than in cardiac tissues. A careful and much more extensive study of the tissue distribution of this protein in the body is needed to look at this problem in more detail.

There are two approaches to establish the possible relationship between the cardiac protein-anthracycline binding and the observed cardiotoxicity. Since the anthracyclines are not endogenous compounds, it is highly unlikely that the receptive protein is a true anthracycline receptor in the sense that it only recognizes the anthracyclines. Presumably, the protein is in the cardiac cells to serve a purpose or it would probably not have survived the vigorous process of evolutionary elimination. One can therefore attempt to identify this protein and define its intrinsic function in vivo. One can further characterize this anthracycline receptive protein by determining its amino acid composition and sequence in the hope that the protein may have been described by other investigators previously. Another approach is to test to see if the protein has an affinity towards endogenous compounds with structures resembling the anthracycline molecule. Alternatively, one can screen this protein for enzymic activities known to be present in the cell cytosol. Very

recently, it was reported that adriamycin inhibited a previously undescribed metmyoglobin reductase isolated from the 100,000 g supernatant of beef heart homogenate (216). This cytosol protein reduced metmyoglobin to its oxygen-carrying form. Additionally, it was shown that adriamycin accelerated the oxidation of oxymyoglobin to metmyoglobin. Thus, adriamycin may conceivably cause a decrease in the intracellular oxygen carrying capacity in the heart by interfering with the oxidation-reduction of myoglobin. Unfortunately, the physicochemical properties of the enzyme were not described in the paper. By this approach a protein such as this may be a candidate for our anthracycline recognizing protein isolated.

If one can find out the function of the receptive protein in the myocytes, one can try to interfere with its function by other means and demonstrate that this will create the same pathophysiological picture observed in the anthracycline induced cardiotoxicity. Alternatively, one might be able to protect the protein or rescue it functionally in some way after anthracycline administration and show that this maneuver can prevent the cardiotoxic action of the drugs.

A less direct approach to addressing the possible relationship between the cardiotoxic effect and the anthracycline-protein interaction is to do correlation studies between the binding affinities of various anthracycline analogs towards the protein, and their *in vivo* cardiotoxicity. The more cardiotoxic compounds would hopefully have a higher affinity towards this protein if the interaction is indeed important for the observed cardiotoxicity. However, this consideration is complicated by pharmacokinetic differences between the analogs.

An *in vitro* assay of cardiotoxicity may help in this respect (e.g. cultured cardiac cells). A binding assay would also have to be developed. The competitive displacement experiments used in this study (p. 76) are qualitative at best and will not be able to distinguish small differences in the binding constants.

This correlation studies approach to the problem is further hindered by several factors. Radio-labelled drugs are not commercially available and have to be synthesized. Although the methodology has been published (217-218), it is very time consuming. The unavailability of anthracycline analogs and the scarcity of *in vivo* cardiomyopathic data, especially in mice, pose additional difficulties. But unless these experiments are performed, the physiological importance of the anthracycline-cardiac protein interaction uncovered by this study remains to be established.

Finally, this report will not be complete without a brief discussion of the potential usage of the receptive materials isolation procedures developed in this study. The procedures are technically very simple; it requires no sophisticated instrumentation and it is relatively inexpensive to be carried out. In this study, the technique was employed to tackle the toxicity problem of the anthracyclines. Using the same technique, one can begin to answer a lot of very important questions concerning the pharmacological actions of the anthracyclines in a very direct way. Some of these questions include:

- Is DNA the only receptor in cancer cells?

- Do cardiac cells and cancer cells possess the same receptor?
- If the answer to the previous question is negative, we can then ask, "Can we design drugs of enhanced specificity around this difference?"
- Do the same receptive materials exist in the heart of different animal species?
- Do all the anthracycline analogs recognize the same receptors in cancer cells, in cardiac cells? In particular, what is the receptor for the N-trifluoroacetyl analogs (see p. 20)?
- Do the metabolites of the anthracyclines recognize the same receptor as the parent compounds?
- Does anthracycline receptor(s) vary qualitatively or quantitatively during different phases of the cell cycle?
- How does resistance towards the anthracyclines develop? Is there any difference in the receptor content in the sensitive cells versus the resistant cells? Are there different types of receptors in the sensitive versus resistant tumor cells?

Since the study of drug receptors and especially those used in chemotherapy is still an infant science, each study such as this not only answers many important questions but opens the door to seeing many new questions.

********** END **********

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