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Preparation of cardiac sarcolemma and investigation of adrenergic and histamine receptor properties using fluorescence spectroscopy

University — Université

University of Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade	Name of Supervisor — Nom du directeur de thèse
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PREPARATION OF CARDIAC SARCOLEMMA AND INVESTIGATION OF ADRENERGIC AND HISTAMINE RECEPTOR PROPERTIES USING FLUORESCENCE SPECTROSCOPY

by

© MARGARET JEAN COLLINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1981

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Margaret Jean Collins

TITLE OF THESIS Preparation of cardiac sarcolemma and investigation of adrenergic andhistamine receptor properties using fluorescence spectorscopy.

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED 1981

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.....
Supervisor

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Date August 14, 1981

TO MY MOTHER AND FATHER

ABSTRACT

Examination of possible thermal changes in histamine receptor properties and the question of α, β-adrenergic receptor thermal interconversion in the heart by fluorescence spectroscopy was the aim of this thesis. Prior to this, purification of sarcolemma containing the histamine H_2 and β-adrenergic receptors was attempted.

Partial purification of sarcolemma from guinea pig ventricular muscle was achieved by successive differential and sucrose gradient centrifugation. Extraction of the contractile apparatus by high salt concentrations was avoided due to its ability to extract membrane proteins and lipids. Enzymes were used to indicate the presence of subcellular membranes. Enrichment of sarcolemma (adenylate cyclase and $(Na^+ + K^+)$-ATPase), possible contamination by outer mitochondrial membranes (MAO and rotenone-insensitive NADH cytochrome c reductase) and sarcoplasmic reticulum (NADPH cytochrome c reductase) and reduced levels of inner mitochondrial membranes (succinate dehydrogenase) were observed in this fraction. The presence of histamine and β-adrenergic receptors was demostrated by stimulation of adenylate cyclase by histamine and INA. Characterization by electron microscopy revealed fragments ranging between 78 and 200 nm in diameter.

Fluorescence spectroscopy on this sarcolemmal fraction was performed using two lipophilic probes, DPH and 12-AS. Histamine H_2 and β-adrenergic agonists and antagonists or the corresponding controls did not affect fluorescence intensity or spectral maxima irrespective of

temperature (10 to 44°C). Thus, drug receptor interaction of the histamine and the adrenergic receptor systems were not observed by this method.

Fluorescence polarization has been employed as a measure of membrane lipid microviscosity. Discontinuous changes in polarization has been observed to correlate with breaks in Arrhenius plots of membrane bound enzymes. However in this study, a continuous change in fluorescence polarization of DPH and 12-AS in the sarcolemmal fraction over the temperature range of 10°C to 44°C was observed. Thus a sudden alteration in membrane viscosity was not detected and could not be responsible for a sudden alteration in receptor properties of either histamine or adrenergic systems in heart.

Introduction of histamine H_2 and β-adrenergic drugs did not affect the fluorescence polarization of DPH and12-AS in the sarcolemmal fraction irrespective of the temperature. No change in polarization was observed with the corresponding controls. Thus interactions of histaminergic and adrenergic drugs with the sarcolemma did not affect the membrane microviscosity.

In summary, drug-receptor interactions of adrenergic and histaminergic agents were not detected by fluorescence intensity, spectra maxima and polarization. A phase transition, a phase separation or a sudden change in membrane lipid fluidity did not occur in this system as observed by fluorescence polarization. If thermal interconversion of the adrenergic receptor or thermal alteration in histamine receptor properties occurs in heart tissue, it appears not to be due to lipid fluidity changes. It is possible that a change in lipid fluidity may be

small and localized to a small area of membrane, the boundary or annular lipids, and may thus be undetected by this biophysical technique.

ACKNOWLEDGEMENTS

I would like to thank:

my supervisor, David A. Cook, for his support and guidance;

Hans Baer for his helpful advice during the adenylate cyclases studies;

Laurie Simonson for his advice concerning enzyme assays;

Christel Krueger for drawing the figures in this thesis;

Gus Duchon for performing the electron microscopy studies and and printing the photographs;

John Phillips for also printing the photographs;

The Canadian Heart Foundation for the financial assistance in the form of a scholarship;

The Alberta Heart Foundation and The Medical Research Council of Canada for financial support in the form of research grants to David Cook;

The Alberta Heritage Foundation for Medical Research for financial support in the form of a research allowance;

Diana, Lou, Rosie, Christel and Francis for making the lab a pleasant place to be;

and all the people who made my stay in Edmonton enjoyable.

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η	= viscosity
AMP	= adenosine-5'-monophosphate
12-AS	= 12-(9-anthroyl)stearic acid
ATP	= adenosine-5'- triphosphate
cyclic AMP, cAMP	= adenosine-3',5'-monophosphate
cyclic GMP, cGMP	= guanosine-3',5'-monophosphate
DCIP	= 2,6-dichlorophenol-indophenol
DPH	= 1,6-diphenyl-1,3,5-hexatriene
DSC	= differential scanning calorimetry
EDTA	= ethylenediamine tetraacetic acid
Em.	= emission wavelength
ESR	= electron spin resonance
Ex.	= excitation wavelength
g	= gravitational acceleration, 9.8 m/sec^2
GTP	= guanosine-5'-triphosphate
HEPES	= N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC	= internal conversion
INA	= isopropylnoradrenaline, isoproterenol
ISC	= intersystem crossing
M	= molar
MAO	= monoamine oxidase
NA	= noradrenaline
NADH	= dihydro(β-)nicotinamide adenine dinucleotide
NADH	= dihydro(β-)nicotinamide adenine dinucleotide phosphate
nm	= nanometer
NMR	= nuclear magnetic resonance
p	= degree of fluorescence polarization
PE	= phenylephrine
POB	= phenoxybenzamine
q	= quantum yield

r = fluorescence anisotropy
RSA = relative specific activity

$$= \frac{\text{specific activity of sample}}{\text{total activity/total protein}}$$

S_0 = ground electronic state
S_n = nth excited singlet electronic state
S.E.M. = standard error of the mean
SR = sarcoplasmic reticulum
S.R. = solvent relaxation

T = absolute temperature (^{o}K)
T_1 = first excited triplet electronic state
T_c = phase transition temperature
THF = tetrahydrofuran
TR = thermal relaxation
Tris = tris(hydroxymethyl)aminomethane

Chapter 1

INTRODUCTION

1.1 The Pharmacological Receptor

Receptors for many hormones and drugs are postulated to be localized on the cell membrane. Supportive evidence for this hypothesis includes the following: in many cases, the biological actions of hormones occur at the plasma membrane; and, many drugs exist, under physiological conditions, in quaternary forms which are unable to pass through the membrane yet are still able to produce the desired biological response. More extensive direct evidence has been accumulated for a number of receptors (for example, acetylcholine, glucagon, insulin, histamine, and noradrenaline receptors). Microionophoresis (Del Castillo and Katz, 1955) has been employed to demonstrate that the presence of acetylcholine was required on the exterior of the cell in order to generate its biological response. Agonists coupled to agarose beads or ferritin have been utilized to show biological responsiveness although the conjugates were unable to penetrate the cell (Atlas et al., 1978; Cuatrecasas, 1974; Jarett and Smith, 1974; Melmon et al., 1972; Weinstein et al., 1973). Treatment with proteolytic enzymes, phospholipases, and other agents which are unlikely to cross cell membranes have been found to decrease the biological responsiveness of the cell with a parallel reduction of agonist binding (Cuatrecasas, 1974). Autoradiography (Atlas et al., 1978; Changeux et al., 1973; Cuatrecasas, 1974; Malhotra, 1978), fluorescent antibody histochemistry (Changeux et al., 1973; Ehrenpreis, 1962), and fluorescent antagonist binding (Atlas and Levitski, 1978) have all been employed in the past to demonstrate the plasma membrane as the site for some pharmacological receptors.

Interaction of hormones or drugs with the plasma membrane receptor has been postulated to result in a conformational change of the receptor which then initiates a series of molecular actions first in the membrane and then, dependent on the receptor system, with the cell to produce the required biological response(s). This drug-receptor interaction has been examined *in vitro* by altering environmental parameters. One such parameter is temperature.

1.1.1 The α, β-Adrenergic Receptor Thermal Interconversion Hypothesis

The effect of temperature on drug-receptor interaction and biological response has been examined in a number of systems. In 1968 and 1973, Kunos and coworkers suggested that receptor properties of the adrenergic system changed with temperature. Above about 24°C, the adrenergic receptor of the frog heart behaved as a β-receptor. It could be blocked by both propranolol and pronethalol, two β-antagonists but not by the α-antagonists, phentolamine and phenoxybenzamine. If the temperature was decreased below 15°C, the receptor took on the characteristics of an α-receptor. The two β-antagonists were no longer effective in blocking the contractile response of adrenaline while the two α-antagonists were now capable of blocking this response. Thus it appeared that β-receptors were predominant above 24°C while α-receptors were predominant below 15°C. Furthermore, when the irreversible α-antagonist, phenoxybenzamine (POB), was first used to block the adrenaline inotropic response at 15°C and then was withdrawn and the temperature raised to 24°C, the POB inhibition remained. It was then suggested that the α- and β-receptors were actually one entity. The adrenergic receptor

was a β-receptor at high temperatures while at lower temperatures the receptor converted into an α-receptor: the temperature thus modulated the configuration of the receptor.

Other studies performed on frog heart (Buckley and Jordan, 1970; Harri, 1973; Kunos and Nickerson, 1976), or rat heart (Amer and Byrne, 1975; Kunos et al., 1973; Kunos and Nickerson, 1977), and on rabbit heart (Endoh et al., 1977) provide supportive evidence for the interconversion hypothesis. In these studies, the order of potency of adrenergic agonists and the potency of α and β antagonists above and below 25°C was examined. At high temperatures the order of agonist potencies was that for the β-receptor, INA > adrenaline > noradrenaline > phenylephrine while at low temperatures the potency appeared as that for the α-receptor, adrenaline > phenylephrine >> INA. Temperature alterations in blocking abilities of propranolol, POB and phentolamine were found to be similar to those observed in frog heart.

Other experimenters have either been unable to reproduce the early work of Kunos and coworkers (1968 and 1973) or have obtained different results. In particular two groups (Buckley and Jordon, 1970; Benfey, 1979a) found that in frog hearts, alkylation of the α adrenergic receptors by POB at low temperatures did not prevent the appearance and functioning of the β-receptor.

Studies on rat heart have produced more confusing findings. Kunos and Nickerson (1977) found that NA, INA, and phenylephrine were all able to produce inotropic responses at 17°C and that on the basis of the potency of the β-antagonist propranolol and the α-antagonists,

phentolamine and phenoxybenzamine, the β-receptors interconvert into α-receptors at lower temperatures. Martinez and McNeill (1977) observed at 17°C, different activities of the adrenergic agonist: NA produced a slight inotropic effect, INA appeared to be ineffective, and phenylephrine had a negative effect. The slight inotropic effect made it quite difficult to examine antagonist potencies. Benfey (1977) found that the rat atrium often did not respond to adrenergic agonists. Another group (Bennett and Kemp, 1978) were unable to keep the rat atrium beating at lower temperatures. Thus, these differences in responsiveness to agonists must be clarified before a proper evaluation of the α, β adrenergic interconversion hypothesis can be satisfactorily undertaken.

The interconversion hypothesis has been examined using the adenylate cyclase system. Agonist interaction with the β-receptor appears to stimulate adenylate cyclase while the α-adrenoreceptor does not have this property. Irrespective of the temperature, stimulation of adenylate cyclase by catecholamines was found to be a β-receptor characteristic in both rat and guinea pig hearts by a number of workers (Benfey et al., 1974; Caron and Lefkowitz, 1974; Martinez and McNeill, 1977). One group (Amer and Byrne, 1975) reported some quite different findings: at 37°C, β-receptors stimulated cAMP production while at 24°C cGMP production occurred. Thus further work on this aspect of α, β interconversion must be performed to clarify the situation.

At present, the α, β-receptor interconversion hypothesis for the adrenergic receptor in heart has not been clearly supported or

refuted on the basis of the accumulated information.

1.1.2 Temperature Dependence of the Histamine Receptor

Another receptor system, the histamine receptor, has been examined for possible thermal interconversion of receptor subtype. It has been suggested that the histamine receptor in the longitudional smooth muscle of the guinea pig ileum showed a partial temperature dependent transition. The histamine response at 37°C was blocked by competitive histamine H_1 antagonists while metiamide, an H_2 receptor antagonist, lacked blocking activity. At 15°C, metiamide was found to inhibit the histamine response while H_1 antagonists had reduced activity or ceased to be competitive (Kenakin et al., 1974).

When phenoxybenzamine, (POB), an irreversible H_1 antagonist as well as an irreversible α-antagonist, was employed at a sufficiently low concentration to yield a shift in the histamine dose response curve at 37° (higher concentrations will produce a shift and a depression of the dose response curve), the tissue cooled to 15°C, the antagonism by POB became noncompetitive and the dose response curve became markedly depressed (Cook et al., 1977). This effect was reversed when the tissue was rewarmed to 37°C. It was proposed that the histamine receptor was altered at lower temperatures, having some properties of both H_1 and H_2 subtypes: that is, it became an "artificially produced intermediate receptor type" (Cook et al., 1977).

The effect of temperature on the histamine receptor was also examined in rabbit renal artery (Cook and Iwanow, 1980). In this tissue, the histamine response was antagonized by the H_1 antagonist, diphenhydramine,

and not by metiamide at 37°C. These same blocking activities were also observed at 15°C. Thus H_1 - H_2 interconversion or altered properties of the histamine receptor did not occur here and may be present only in some tissues.

1.1.3 Temperature Effects of Membrane Bound Enzymes

If a change in receptor properties or possibly an interconversion of receptor subtypes occurs after alteration of the temperature, it may be due to a change in receptor conformation and/or to a change in receptor-lipid interaction. These ideas are similar to those used as explanations of observations with lipid-dependent enzymes. Not only do a number of enzymes, such as mitochondrial ATPase (Cunningham and George, 1975; Dabbeni-Sala et al., 1974) and β-hydroxybutyrate dehydrogenase (Gazzotti et al., 1975; Grover et al., 1975), plasma membrane ($Na^+ + K^+$)-ATPase (Kimelberg and Papahadjopoulos, 1974; Tanaka et al., 1971; Walker and Wheller, 1975), Ca^{2+}-ATPase of the sarcoplasmic reticulum (Fiehn and Hasselback, 1970; Martonosi et al., 1971; Warren et al., 1974) and microsomal NADH-cytochrome b_5 reductase (Rogers and Strittmatter, 1973) have certain lipid requirements for their activities but also Arrhenius plots for many membrane bound enzymes show sharp discontinuities - that is, when the parameter log activity is plotted versus the inverse of temperature, the plot consists of two intersecting straight lines (Charnock et al., 1971; Charnock et al., 1973; Farías et al., 1975; Kimelberg and Papahadjopoulous, 1974; Lenaz, 1979; Sandermann, 1978) rather than a single straight line found for most soluble enzymes (Sandermann, 1978). In some cases, a correlation between the discontinuity in the Arrhenius

plot of the purified enzyme and the phase transition temperature of its membrane lipids (this temperature is the "critical temperature", T_c, where the lipids exist in both the liquid-crystalline phase and the gel phase; it is similar to that of the freezing point of a compound) has been observed (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1974; Overath et al., 1975; Raison et al., 1971). In other cases, calorimetric phase transitions were not found (Hackenbrock et al., 1976; Lee et al., 1974; Martonosi, 1974) yet spectroscopic methods such as ESR (electron spin resonance) (Inesi et al., 1973; Lee et al., 1974; Raison and McMurchie, 1974; Wu and McConnell, 1975; Wunderlich et al., 1975), NMR (nuclear magnetic resonance) (Davis et al., 1976), and fluorescence (Bashford et al., 1976; Wunderlich et al., 1975), showed changes in membrane fluidity corresponding to the discontinuities in the Arrhenius plots. These breaks in Arrhenius plots have been interpreted as the result of a phase transition of membrane lipids (Morrisett et al., 1975; Overath et al., 1975; Sandermann, 1978). Another interpretation which has been suggested is these thermal discontinuities are caused by a phase separation of membrane lipids (Linden et al., 1973; Raison and McMurchie, 1974; Wu and McConnell, 1973; Wunderlich et al., 1975). More recently they have been explained in terms of a change in the fluidity of the lipids immediately surrounding the enzyme, that is, the annular or boundary lipids, with a corresponding alteration in lipid-protein interaction which then affects enzymatic properties (Charnock and Bashford, 1975; Lenaz, 1979; Marcelja and Wolfe, 1979).

Even temperature dependent alterations in permeability properties

(Papahadjopoulos et al., 1973; Schreier-Muccillo et al., 1976), transport functions (Linden et al., 1973; Shechter et al., 1972; Tsong, 1975), hormone binding (Bashford et al., 1975a; Postel-Viney et al., 1974), and hormone stimulation of adenylate cyclase (Houslay et al., 1976) have been demonstrated. Changes in allosteric behaviour (as measured by the Hill coefficient) for some membrane bound enzymes appear when the membrane fluidity is altered (Farías et al., 1975).

Thus if Kunos' idea of the α - β adrenergic thermal interconversion or some alteration in adrenergic receptor properties in cardiac tissue is found to be correct and the change in histamine receptor properties occurs in some tissues, perhaps these are a result of membrane fluidity changes and/or receptor-lipid interactions. It was then of interest to examine the α, β thermal interconversion hypothesis in heart by observing fluidity changes of the membrane lipids by the use of a biophysical technique. Possible alterations in histamine receptor properties in cardiac tissue (an H_2 system here at 37°C) were also to be investigated. To do this it was necessary to obtain partially purified sarcolemma containing both histamine receptors and β-adrenergic receptors. Once this was accomplished, this sarcolemma would be tested for any possible discontinuous changes in membrane fluidity as a function of temperature. The effect of drugs acting on the histamine receptor and the adrenergic receptor would be ascertained to determine whether any drug-receptor interaction in some way altered local membrane fluidity and if there was a corresponding temperature dependence possibly related to a change in receptor properties.

A biophysical technique used to examine fluidity changes was

required. Four possible methods were DSC (differential scanning calorimetry), ESR (electron spin resonance), fluorescence spectroscopy, and NMR (nuclear magnetic resonance). NMR was rejected due to its low sensitivity (Badley, 1976). DSC was eliminated since it would only detect gross changes in lipid states such as gel - liquid - crystalline phase transition and bilayer - hexagonal packing (Lenaz, 1979). Both fluorescence and ESR are similar in their advantages and disadvantages, although fluorescence appears to be more sensitive (Badley, 1976; Radda, 1971; Radda and Vanderkooi, 1972). For this reason and the availability of the instrumentation, fluorescence spectroscopy was chosen as the biophysical technique to be employed in this study.

1.2 Fluorescence Spectroscopy

1.21. General

The theory of fluorescence spectroscopy as it applies to biomembranes has been extensively discussed and there are many good indepth reviews (Azzi, 1975; Badley, 1976; Guilbault, 1973; Radda, 1975a; Radda and Vanderkooi, 1972; Skoog and West, 1971) on the subject. A brief summary of the necessary information will be given here.

The phenomenon of fluorescence involved the absorption of light by a molecule followed by release, or emission, of light of a different energy and therefore, at a different wavelength. All molecules are characterized by a set of molecular orbitals with their corresponding allowed electronic energy levels. Superimposed on each electronic energy level is a series of closely spaced vibrational energy levels (Figure 1.1). Quantum mechanical theory states that a molecule is only allowed certain electronic energies and certain vibrational energies which are defined by molecular orbital wavefunctions. At room temperature, most molecules are in their ground electronic state (S_0) and in its corresponding lowest vibrational level (V = 0). If the molecule is exposed to light, it can undergo a transition to a higher energy level. The molecule absorbs a fixed quantum of light, the energy ($E = h\nu$) of which must be equal to the energy difference between the ground state, S_0, and the excited state (a different electronic level and possibly a different vibrational level). The excited state may be S_1, S_2, etc., that is, the first excited singlet state, the second excited singlet state, etc.

Before the emission of light, or flourescence, occurs, the

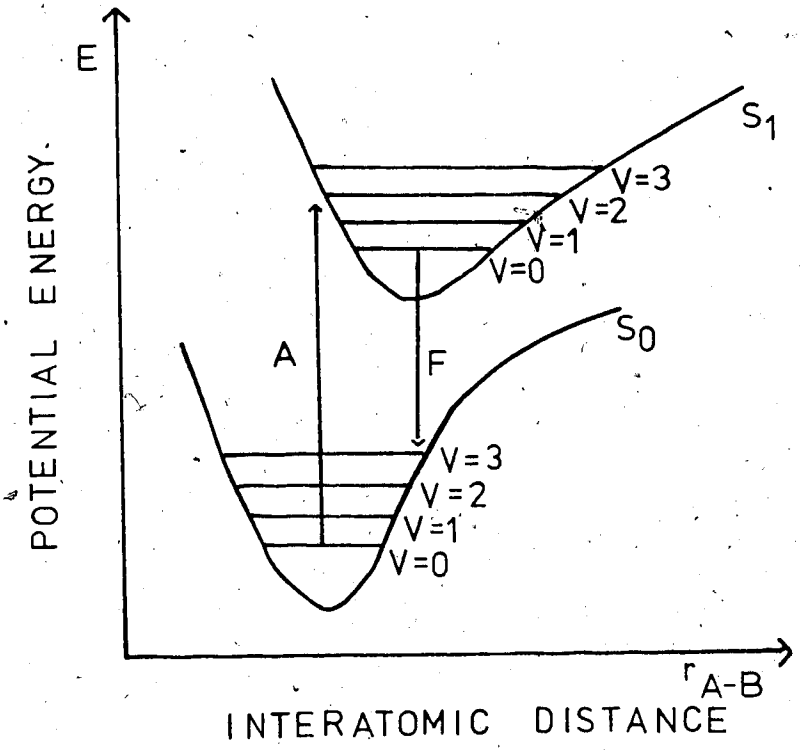


Figure 1.1. Potential energy profiles for ground state (S_2) and first excited state (S_1). A = absorption of light; F = emission of light, or fluorescence; V_n = n^{th} vibrational level.

molecule undergoes a number of processes (Figure 1.2). Some of the absorbed energy is rapidly lost through exchange with solvent molecules (thermal relaxation) so that the lowest vibrational level (V = 0) of the excited singlet state is reached. Also, transition from higher excited states (S_2, S_3, etc.) takes place via internal conversion (radiationless process) to the lowest vibration level of S_1. The molecule may now return to the ground electronic state, S_0, with the corresponding release of the excess energy in the form of light (fluorescence) which equals the energy difference between S_1 and S_0.

A number of processes may compete with this fluorescence. Loss of the excess energy may occur via internal conversion processes with the energy dissipation to the surrounding solvation shell. Another competing process is intersystems crossing to a lower lying triplet state (eg. T_1). From this excited triplet state excess energy is lost either via non-radiative processes or via light emission, known as phosphorescence. Because of the large difference in rate constants, the non-radiative processes are much more significant.

A measure often employed to quantify the importance of processes competing with fluorescence is the quantum yield. The quantum yield is defined as:

$$q = \frac{\text{quanta emitted}}{\text{quanta absorbed}} = \frac{k_f}{k_f + k_p + \Sigma k_i}$$ where k_f is the rate constant for fluorescence, k_p is the rate constant for phosphorescence, and Σk_i denotes the sum of rate constants for all non-radiative processes. Thus the use of a molecule as a fluorophore will depend on the value of q.

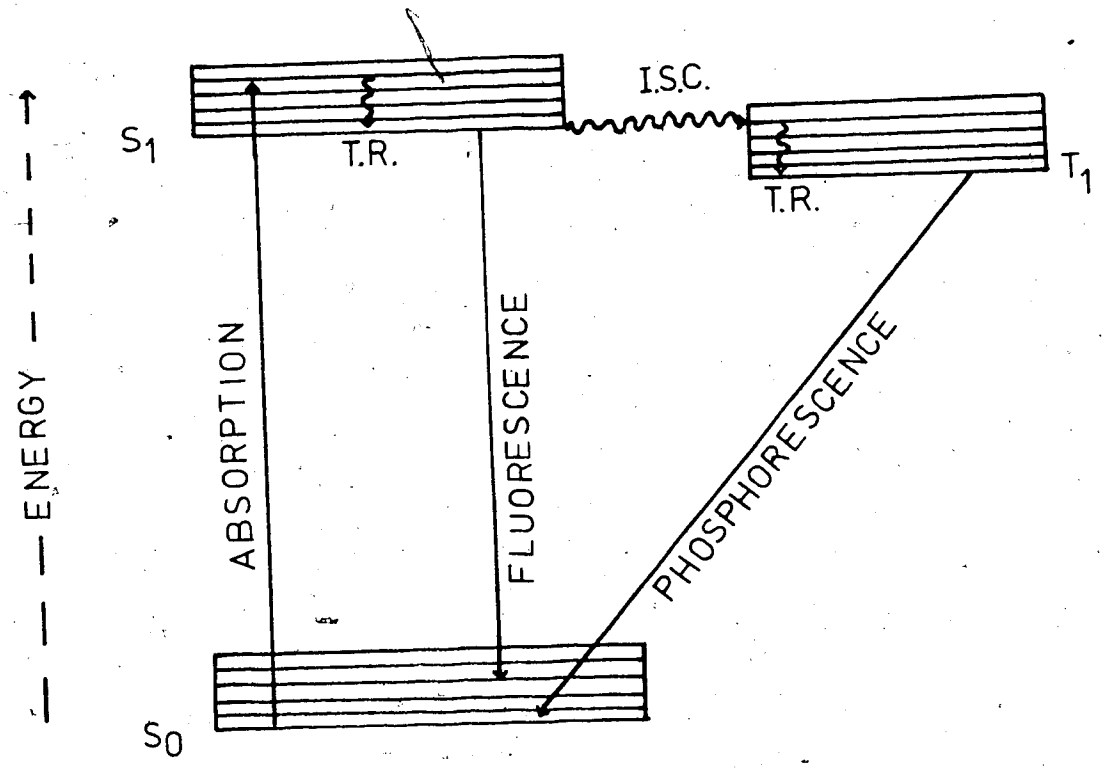


Figure 1.2. Simplified Jablonski diagram for energy levels in electronic transitions. S_0 = ground state; S_1 = first excited singlet state; T_1 = first triplet state; T.R. = thermal relaxation; I.S.C. = inter-system crossing.

The closer the value is to unity, the better the fluorophore.

The fluorescence emission is observed at higher wavelengths than that of the exciting light. The difference in wavelengths is referred to as Stokes Shift. It can be explained by examining the fluorescent process. Prior to emission of light by the excited molecule, some of the absorbed energy is lost to the thermal conversion and non-radiative processes which allow the excited molecule to relax to the lowest vibrational energy level of the first excited singlet state. The molecule now has less absorbed energy and on conversion from S_1 to S_0 will release this reduced energy and consequently the light will be emitted at longer wavelengths.

The immediate environment of the fluorophore (a molecule which can undergo fluorescent emission) will influence the energy levels of the ground state and the excited state. The Stokes Shift and the wavelength maxima of excitation and emission spectra can be altered. The nature of the solvent (polarity and viscosity), the pH, the presence of quenchers and the temperature all affect the fluorescence of a molecule. Solvent-induced spectral shifts due to polarity will be examined here to demonstrate how the environment can affect the fluorescent properties. In a nonpolarhydrocarbon solvent, a fluorophore will have a certain fluorescent maximum. The energy difference between the absorption maximum and the emission maximum will determine the magnitude of the shift.

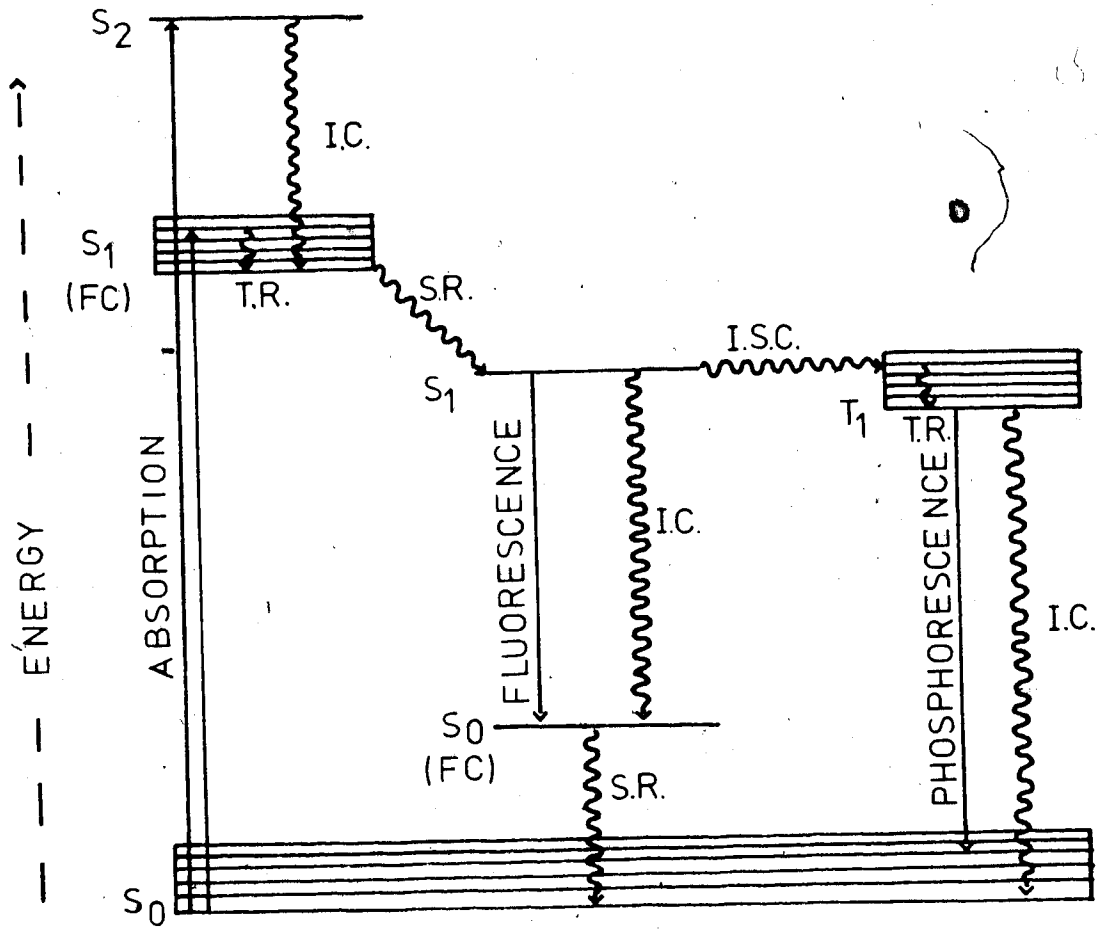
If the excited state of the fluorophore is more polar than the ground state, as in the case of most polar molecules, then an increase

in solvent polarity stabilizes the excited state to a greater extent than the ground state. The absorption maximum and emission maximum are shifted to lower energies and therefore to longer wavelengths (Figure 1.3). Since the energy of S_1 is reduced much more than S_0, the fluorescence maximum will be more red-shifted than the absorption maximum. This greater red-shift occurs because during excitation of the fluorophore, the surrounding solvent shell undergoes reorientation within about 10^{-11} to 10^{-12} seconds. The excited fluorophore is then in an "equilibrium" excited state where the solvent configuration is optimal for the geometry and the electron distribution of the molecule. Upon emission the molecule returns to the ground state where again the solvation shell rearranges and thus reduces the energy.

The viscosity of the solvent will also affect the fluorescence properties of a fluorophore. With increasing viscosity, the solvent relaxation rate may become too slow to be completed before emission occurs. The equilibrium excited state is then not reached and thus the emission is shifted to a higher energy and lower wavelength. The quantum yield of the fluorophore is increased. Here an increase in solvent viscosity reduces the frequency of collisions and thus decreases the probability of external non-radiative conversion.

There are a number of fluorescence parameters which can be measured: fluorescence intensity, excitation and emission spectra, quantum yield, lifetime and polarization. All these parameters will be affected differently by different environmental influences. In the case of fluidity (the inverse of viscosity), which is the environmental

Figure 1.3. Jablonski diagram for energy levels in electronic transitions. S_0 = ground state; S_1 = first excited singlet state; S_2 = second excited singlet state; T_1 = first excited triplet state; I.C. = internal conversion; T.R. = thermal relaxation; S.R. = solvent relaxation; and I.S.C. = intersystem crossing. F.C. denotes Franck-Condon states which are states not in equilibrium with the solvent. Other states are in equilibrium with the solvent.


S2
I.C.
S1
(FC)
T.R.
S.R.
S1
I.S.C.
T1
T.R.
ABSORPTION
FLUORESCENCE
I.C.
PHOSPHORESCENCE
I.C.
S0
(FC)
S.R.
S0
ENERGY

influence to be monitored by the fluorophore in this study, static and nanosecond polarization has been employed extensively. To a lesser extent, fluorescence intensity has been used to study fluidity changes in phospholipid membranes. A brief description of fluorescence polarization will be given here; followed by its application to biomembranes.

1.2.2 Fluorescence Polarization

One particular fluorescence parameter, fluorescence polarization, has been employed extensively over the last few years to examine the mobility of the fluorophore (or the molecule to which the fluorophore is covalently attached), its orientation and the microviscosity of the environment in membranes and other biological systems (Shinitzky and Barenholz, 1978). Polarization of fluorescence is measured by monitoring the intensity of emitted light at right angles to the excitation beam of plane-polarized monochromatic light. The degree of polarization, p, and a commonly employed alternate parameter, fluorescence anisotropy, r, are defined (Azzi, 1975; Steinberg, 1975) as follows:

$$p = \frac{I_{11} - I_1}{I_{11} + I_1}$$

and

$$r = \frac{I_{11} - I_1}{I_{11} + 2I_1}$$

where I_{11} and I_1 are the intensities of emitted light parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. The degree of fluorescence polarization and fluorescence anisotropy are interrelated:

$$p = \frac{3r}{2 + r}$$

and

$$r = \frac{2p}{3 - p}$$

Total fluorescence intensity, F, is equal to $I_{11} + 2I_1$ which is the denominator in the equation defining r.

Both fluorescence anisotropy and degree of fluorescence polarization are confined to a certain range of values. The theoretical derivation for these constraints is well described by Badley (1976) and Shinitzky and Barenholz (1978). P may take on the values from -1/3 to +1/2 while r is limited to the range -1/5 to +2/5. These outside limits are derived for an ideal fluorophore in a random rigid solution.

If molecular motion can occur on a time scale faster than the lifetime of the excited state, then complete randomization will result in values of p = r = 0. If molecular rotations occur on the same time scale, partial depolarization will occur. The observed degree of polarization can be described by the Perrin equation (Badley, 1976):

$$\left\{\frac{1}{p} - \frac{1}{3}\right\} = \left\{\frac{1}{p_0} - \frac{1}{3}\right\}\left\{1 + \frac{RT\tau}{\eta V}\right\}$$

where p is the observed polarization, p_0 is a constant and is the value of p extrapolated to zero temperature and infinite viscosity, R is the gas constant, T is the absolute temperature ($^{\circ}K$), τ is the lifetime of the excited state, η is the viscosity of the solvent shell (the microviscosity) and V is the effective molecular volume.

The Perrin equation can also be expressed in terms of anisotropy,

r (Azzi, 1975; Shinitzky and Barenholz, 1974):

$$\frac{\frac{1}{p} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = \frac{r_0}{r} = 1 + \frac{C(r)T\tau}{\eta}$$

where p, p_0, T, τ and η are defined above, r is the observed anisotropy, r_0 is a constant which is the value of r extrapolated to zero temperature and infinite viscosity and C(r) is a term which depicts the rotational relaxation time of the solute and varies with each observed anisotropy value. On examination of the temperature dependence of fluorescence polarization of a commonly employed probe, DPH, for both liposomes and membrane systems, a plot of $\left\{\frac{r_0}{r} - 1\right\}^{-1}$ versus produced a straight line with a value of 2.4 poise for the inverse of the slope, C(r)·T·τ (Shinitzky and Barenholz, 1978). A decrease in T will be compensated by an increase in τ and C(r) and the term C(r)·T·τ remains approximately constant. Thus, fluorescence anisotropy and degree of polarization can be used as a measure of the microviscosity surrounding the fluorophore DPH.

The use of fluorescence polarization as a measure of membrane fluidity was initially tested in liposomes. Polarization of DPH in sonicated DMPC (dimyrestoyl phosphatidylcholine) unilamellar vesicles was found to change markedly (Andrich and Vanderkooi, 1976; Lentz et al., 1976a) through the phase transition as observed by both DSC (Hinz and Sturtevant, 1972) and ESR (Shimshick and McConnell, 1973). Results obtained with other monolipid systems are also in agreement with those of ESR and DSC (Hinz and Sturtevant, 1972; Lentz and Barenholz, 1974;

Lentz et al., 1976a; Shimshick and McConnell, 1972; Suurkuusk et al., 1976). Phase transitions and phase separations occurring in liposomes containing two or more lipid types are also detected by fluorescence polarization and the temperatures correspond to those found using DSC and ESR (Andrich and Vanderkooi, 1976; Jacobson and Papahadjopoulos, 1975; Lentz et al., 1976b; Shimshick and McConnell, 1973; Shinitzky et al., 1979).

Fluorescence spectroscopy has also been used to measure microviscosity in biological membranes. The effects of additional cholesterol, divalent cations, cellular fusion, and alteration in the growth temperature on the microviscosity have been examined (Bashford et al., 1975a; Cogan et al., 1973; Martin and Thompson, 1978; Shinitzky and Inbar, 1974; Vanderkooi et al., 1974b; Weidekamm et al., 1976). Again similar results were observed to those obtained using DSC. Microviscosity, as measured by fluorescence polarization, was examined for a number of enzymes which show "bent" Arrhenius plots. $(Na^+ + K^+)$-ATPase (Charnock and Bashford, 1975) and NADH oxidase (Radda, 1975b) are two such enzymes. For both enzyme systems, the lipid bilayers were found to undergo a sudden change in microviscosity which corresponded to the temperature at which there was a discontinuity in the Arrhenius plot for each enzyme. The thermal discontinuity in binding of thyroid stimulating hormone also appeared to correlate with fluorescence polarization changes (Radda, 1975c).

In particular, there are a few studies on drug effects on biological membranes. Such actions as insulin on liver membranes (Luly and

Shinitzky, 1979), β-antagonists on human erythrocyte membranes (Akiyama and Igisu, 1979), muscarinic agents on plasma membranes from intestinal smooth muscle (Spero, 1978), thyroid stimulating hormone on thyroid membranes (Bashford et al., 1975a) and anaesthetics on various membranes (Elfedink, 1977; Radda, 1975; Rooney et al., 1979) have been observed.

Thus it seems that fluorescence spectroscopy, and in particular fluorescence polarization, are useful tools in the study of membrane microviscosity and fluidity.

The choice of fluorescent probes is important. There are many available compounds. A list can be obtained from the reviews by Badley (1976), Radda (1975a) and Shinitzky and Barenholz (1978). Since changes in membrane fluidity were to be monitored, probes which were located deep in the lipid hydrocarbon core were chosen. Also, polarization values, and thus microviscosity, alter to a greater extent as the fluorescent probe is positioned deeper in the lipid bilayer (Radda, 1975a). Two probes, DPH (1,6-diphenyl-1,3,5-hexatriene) and 12-AS (12-(9-anthroyl) stearic acid), were chosen. Both these probes have been shown to be located deep in the hydrocarbon core of the bilayer by the use of NMR and electron diffraction (Jacobson and Papahadjopoulos, 1975; Lentz et al., 1976; Lesslauer et al., 1972; Podo and Blasie, 1977; Shinitzky and Barenholz, 1974; Shinitzky and Inbar, 1974; Vanderkooi et al., 1974).

In particular, 12-AS is said to be located in the bilayer with its free carboxyl group in line with the carboxyl moiety of the fatty acyl chains of the phospholipids and the fluorescent moiety (the anthroyl

ring) positioned near the terminal methyl group of the fatty acyl chains (Lesslauer et al., 1972; Radda, 1975a). A schematic representation of this concept is shown in Figure 1.4.

DPH, is also thought to be present deep in the lipid bilayer. Its localization is also depicted in Figure 1.4. It is suggested that it lies parallel to the fatty acyl chains of the phospholipids without exposure of any part to the head group and aqueous interface. It is also thought to be present perpendicular to the phospholipids, but in close proximity to the terminal methyl group of these lipids.

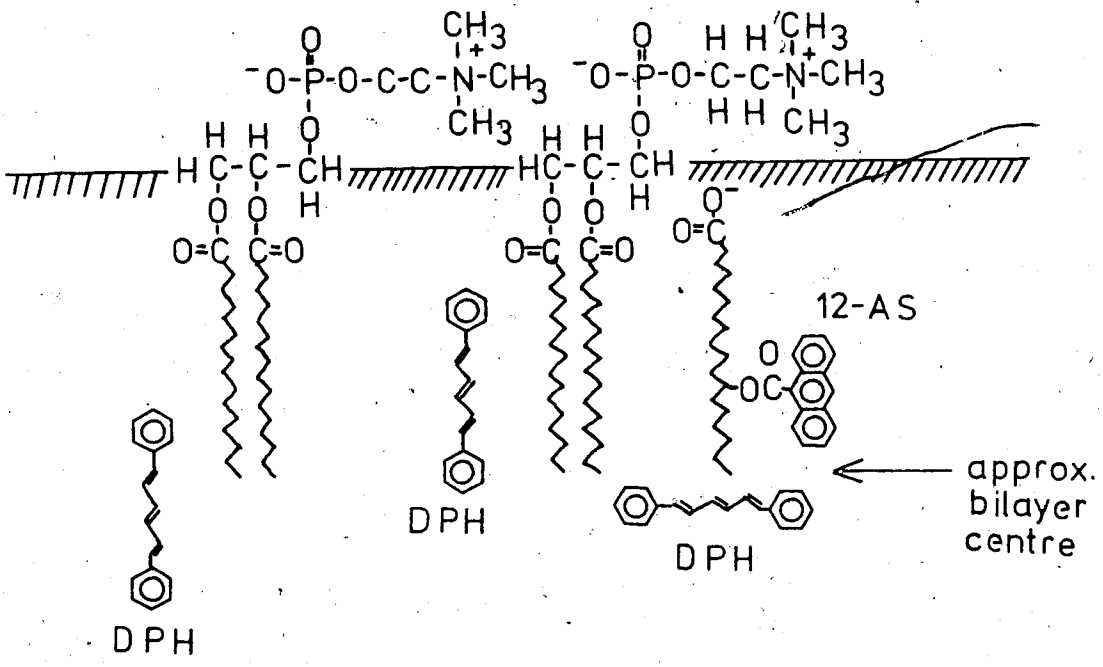


Figure 1.4. Suggested locations for the fluorescent probes 12-AS and DPH in phospholipid bilayers.

1.3 Summary of Objectives

Possible interconversion of receptor subtype or possible alteration in drug receptor properties of the adrenergic and histamine receptor systems in the heart and the role of the surrounding lipids in any change was to be examined using a biophysical technique. In order to do this, it was necessary to:

1. partially purify sarcolemma containing both histamine H_2 and β-adrenergic receptors.
2. examine whether the membrane fluidity of the fraction containing both receptors suddenly changes as the temperature is lowered. Membrane fluidity was to be measured by fluorescence polarization of two probes, 12-AS and DPH.
3. ascertain any effects of histaminergic and adrenergic drugs on membrane fluidity over the temperature range $10^{o}C$ to $44^{o}C$.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Adenosine-5'-diphosphate (ADP: sodium salt), albumin (bovine serum), DL-12-(9-anthroyl)-stearic acid (12-AS), cytochrome c (horse heart, type III), 2,6-dichlorophenol-indophenol (sodium salt, grade I), diphenhydramine (hydrochloride), 1,6-diphenyl-1,3,5-hexatriene (DPH), glycylglycine, histamine (dihydrochloride salt), L-histidine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), DL-isoproterenol (INA: hydrochloride salt), kynuramine (dihydrobromide salt), L-lactate dehydrogenase (rabbit muscle, type II), magnesium chloride (hexahydrate), magnesium sulphate (heptahydrate), (-) noradrenaline (bitartrate salt), β-nicotinamide adenine dinucleotide-reduced form (NADH: cyclohexylamine salt and disodium salt), β-nicotinamide adenine dinucleotide phosphate-reduced form (NADPH: tetrasodium salt), p-nitrophenylphosphate (disodium salt), ouabain (octahydrate), phenylephrine (hydrochloride), phospho-(enol)pyruvate (PEP: tri-monocyclohexylammonium salt), pyruvate kinase (rabbit muscle, type II), rotenone, succinic acid (disodium salt, hexa-hydrate), sucrose (grade II), and tris(hydroxymethyl)aminomethane (Tris) were all purchased from Sigma Chemical Co.

Acetone (Spectranalyzed®), calcium chloride, dextrose (anhydrous), ethylenediamine tetraacetic acid (H_4EDTA), hydrochloric acid, lithium chloride, magnesium sulphate (anhydrous), methanol (Spectranalyzed®), perchloric acid (70%), phenol reagent solution (2N, Folin-Ciocalteau), potassium hydroxide, potassium phosphate monobasic (KH_2PO_4), sodium bi-carbonate, sodium chloride, sodium fluoride, and toluene were obtained from Fisher Scientific Co. Ltd.

Adenosine -3',5'-monophosphate (cyclic AMP or cAMP), adenosine -5'-monophosphate (AMP: disodium salt), adenosine -5' triphosphate (ATP: disodium salt), creatine kinase (rabbit muscle), creatine phosphate, and guanosine 5'-triphosphate (GTP: disodium salt) were provided by Boehringer Mannheim.

The following chemicals were also used: α-^{32}P-ATP (solium salt; ICN Chemicals), chlorpheniramine (maleate salt; Schwartz/Mann), cimetidine (Smith, Kline, and French), cupric sulphate (T.J. Baker Chemical Co.), 1,4-bis(2-(4-methyl-5-phenyloxazoyl)) benzene (dimethyl POPOP: Packard Instrument Company, Inc.), 2,5-diphenyloxazole (PPO; Koch-Light Laboratories Ltd.), Dowex 50W® (Bio Rad Laboratories), 4-hydroxyquinoline (Aldrich Chemical Company), metiamide (Smith, Kline, and French), 4-nitrophenol (Aldrich Chemical Company), phentolamine (mesylate salt; Ciba), potassium chloride (Analar®; BDH Chemicals Ltd.), potassium cyanide (Analar®; BDH Chemicals Ltd.), propranolol (Ayerst), sodium carbonate (anydrous; J.T. Baker Chemical Co.), sodium hydroxide (Matheson, Coleman, and Bel), and tetrahydrofuran (Tetrachemical Laboratories Ltd.).

2.2 Methods

2.2.1 Preparation of Sarcolemma Fraction Containing β-adrenergic and Histamine Receptors

Purification of the histamine receptor from heart has not been attempted to date and very little work has been done on cardiac β-adrenoceptor purification. The literature on cardiac plasma membrane purification and isolation (Alstyne et al., 1979; Bers, 1979; Besch et al., 1976, 1977; Evans, 1978; Hui et al., 1975, 1976; Jones et al., 1979; Kidwai, 1974; Kidwai et al., 1971; Misselwitz et al., 1979; Paris et al., 1977; Pitts, 1979; Saccomani et al., 1974; Scarpa and Williamson, 1974; Schimmel et al., 1977; St. Louis and Sulakhe, 1976; Tada et al., 1972; Wang et al., 1977; Williams et al., 1977) was examined. Common to most proceedures are the use of isotonic sucrose and EDTA in the buffer and the combination of differential and density gradient centrifugation for separation of fractions. The pH varied from 6.8 to 8.1. Furthermore, the speed of centrifugation as well as the gradient parameters varied from method to method. These factors were varied systematically and an experimental proceedure established; this is described below.

Six male guinea pigs, each weighing 350 to 450 g, were injected intraperitioneally with heparin (10,000 USP units). Twelve minutes after injection, the guinea pigs were killed by a blow to the head and exsanguinated. The hearts were excised and placed in cold oxygenated Krebs solution (Edinburgh staff, 1974). All subsequent steps were performed at 0 to 4°C. The hearts were cleaned of connective tissue and blood

vessels, the atria were removed and only the ventricles were used. The ventricles were then suspended in a working buffer (30 mM L-histidine, pH 7.5, 5 mM EDTA, 250 mM sucrose) to make up a 10% v/v suspension (approximately 7% w/v). They were minced with scissors, homogenized for three five-second intervals at half maximal speed using a Polytron Model PT20, and then filtered through four layers of cheesecloth to remove connective tissue and other debris. This homogenate (referred to hereafter as 'homogenate') was centrifuged at 1,000 g_{max} for twenty minutes using SS-34 fixed angle rotor in a Sorvall RC2-B preparative centrifuge. The supernatant was saved for later use. The pellet was suspended in 45-50 mls of working buffer, rehomogenized again for three five-minute intervals at half maximal speed, and then refiltered through four layers of cheesecloth. After filtration, the suspension was recentrifuged at 1,000 g_{max} for twenty minutes. The pellet obtained here was denoted "1,000 g pellet" and suspended in 40 mls of storage buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose). The supernatant obtained at this step was pooled with the previous supernatant and then centrifuged at 9,000 g_{max} for thirty minutes. The resultant pellet was labelled "9,000 g pellet" and was suspended in 20 mls of storage buffer. The resultant supernatant was centrifuged at 110,000 g_{ave} for one hour in a Beckman 60 Ti fixed angle rotor using a Beckman B-40 ultracentrifuge. The resultant supernatant was labelled "supernatant". The pellet was suspended in a minimal volume (approximately 2 mls) of storage buffer and then loaded onto a continuous sucrose gradient (15 to 55% w/w) containing 20 mM Tris, pH 7.4, and 1 mM EDTA. The gradient was centrifuged

at 103,000 g_{ave} for 180 minutes in a Beckman SW-41 swinging bucket rotor, using a Beckman L3-40 ultracentrifuge. Five fractions were collected at 12%, 14%, 26%, 34%, and 40% w/w sucrose and labelled F1, F2, F3, F4, and F5 respectively. Fractions were diluted to approximately 8% sucrose, except when used for adenylate cyclase and fluorescence measurements where they were used undiluted. See Figure 2.1 for a schematic representation of the proceedure.

2.2.2 Enzymatic Characterization

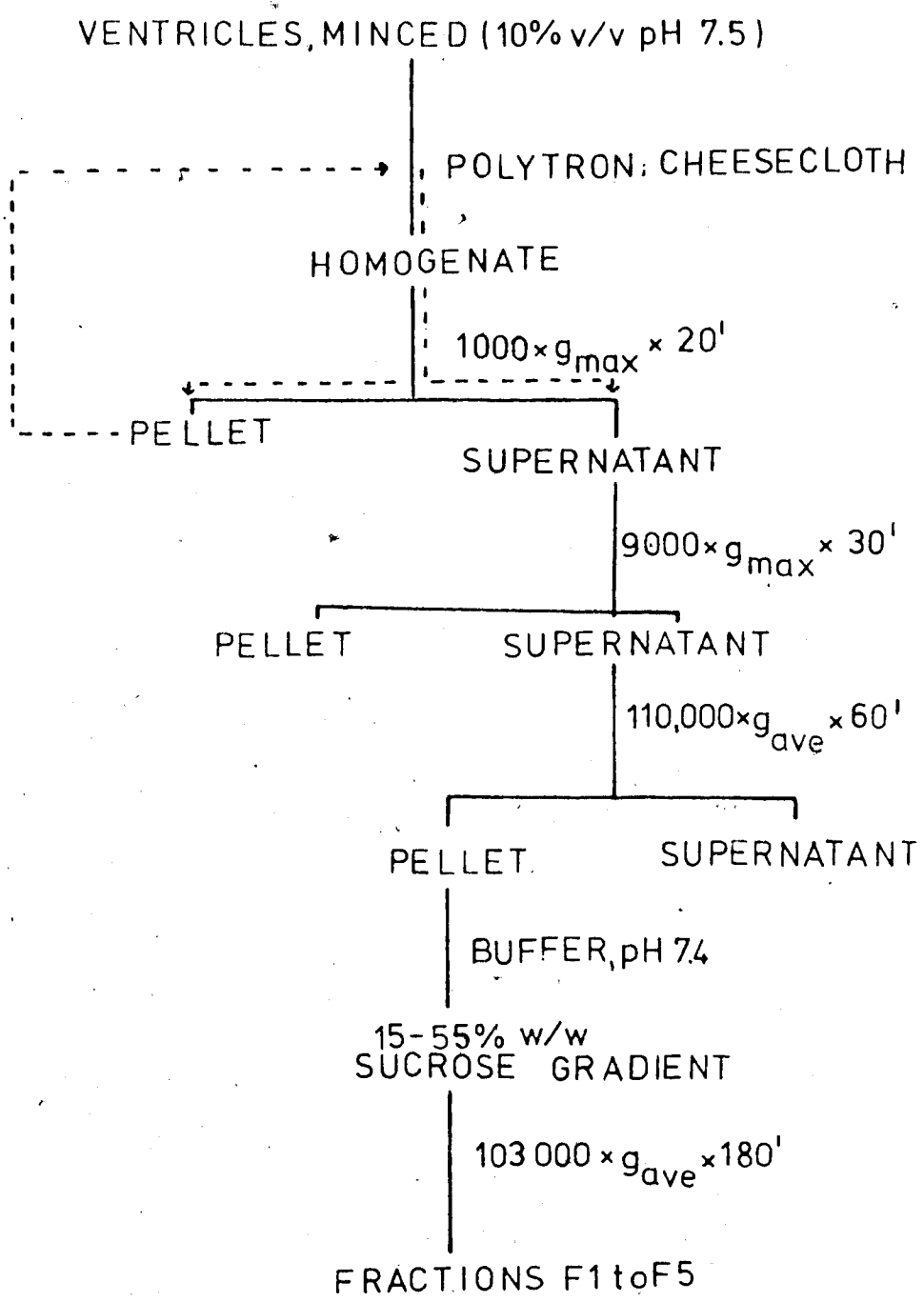
a) Protein Determination

Protein concentration was determined by the Lowry method (Lowry et al., 1951). Bovine serum albumin was employed as a standard. A standard curve was constructed each time with no less than 10 points.

b) Adenylate Cyclase

Adenylate cyclase levels were measured by the Polyimine cellulose method of Baer 9Baer, 1975). Reaction conditions for basal activity were: 25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.5, 5 mM MgCl, 0.2 mg/ml creatine kinase, 20 mM ceeatine phosphate, 1 mM cyclic AMP, 0.1 mM α-^{32}P-ATP. The reaction was stopped by addition of nonradioactive ATP, AMP, and cAMP (final concentrations were approximately 3.4 mM for each compound) and then by cooling on ice. 10 mM sodium fluoride was added to measure fluoride stimulation of adenylate cyclase. For both histamine (10^{-4} M) and isoproterenol (10^{-5} M) stimulation of adenylate cyclase, GTP (10^{-5} M) was found to be required for optimal activity. This concentration of GTP did not cause any stimulation of adenylate cyclase. Incubation time was 25 minutes, except in the case

Figure 2.1. Schematic representation of the isolation proceedure for cardiac sarcolemma containing histamine and β-adrenergic receptors. See text for further details.


VENTRICLES, MINCED (10% v/v pH 7.5)
POLYTRON; CHEESECLOTH
HOMOGENATE
1000×g_max × 20'
PELLET
SUPERNATANT
9000×g_max × 30'
PELLET
SUPERNATANT
110,000×g_ave × 60'
PELLET
SUPERNATANT
BUFFER, pH 7.4
15-55% w/w
SUCROSE GRADIENT
103 000 × g_ave × 180'
FRACTIONS F1 to F5

of F3, F4, and 9,000 g samples where the incubation time was decreased to 5 minutes for optimal assay conditions. Samples were stored, undiluted, in liquid nitrogen and were assayed immediately upon thawing.

The scintillation medium employed for radioactive counting was toluene based (4 g PPO and 0.2 g dimethyl POPOP per litre of toluene) and the samples were counted on a Beckman LS-330 scintillation counter.

c) $(Na^+ + K^+)$-ATPase

$(Na^+ + K^+)$-ATPase was measured according to the method of Charnock et al. (1977). In this spectrophotometric method, $(Na^+ + K^+)$-ATPase activity is determined indirectly via enzymatic coupling; the actual recorded absorbance change is due to the oxidation of NADH to NAD^+. The reaction medium consisted of 100 mM glycylglycine (adjusted with Tris to pH 7.6), 2mM $MgSO_4$, 0.2 mM EDTA, 20 mM KCl, 250 mM sucrose, 14.3 U/ml Lactic dehydrogenase, 23.2 U/ml pyruvate kinase, 3.14 mM phospho(enol) pyruvate, 0.28 mM NADH, 80 mM NaCl, and 1.5 mM ATP. This medium measured total ATPase ($(Na^+ + K^+)$-ATPase plus Mg^{2+}-ATPase values. The ATP was introduced into the heated (37^oC) cuvettes immediately prior to the initiation of the assay by addition of the sample. The reaction was observed at 340 mM on a Gilford Model 2400 recording spectrophotometer. The concentration of oxidized NADH, and thus indirectly the concentration of hydrolyzed ATP, was calculated using a molar extinction coefficient of $6.22 \times 10^{+3}\ M^{-1}$. $(Na^+ + K^+)$-ATPase levels were then calculated by subtracting the Mg^{2+}-ATPase levels from the total ATPase levels.

d) Alkaline Phosphatase

Alkaline phosphatase activity was examined by a modification of

the mthod of Perkarthy et al. (1972). The formation of p-nitrophenol at pH 10.3 was monitored at 410 nm on a Gilford Model 222 recording spectrophotometer. The assay medium contained 5 mM p-nitrophenol-phosphate, 5 mM $MgCl_2$, 200 mM Tris buffer, pH 10.3. Upon addition of the membrane enzyme sample, the assay system was incubated at 37^{o} for 1 hour. p-Nitrophenol concentrations were calculated from a standard curve obeying Beer's Law in the range of 1 μM to 100 μM. Nonenzymatic degradation was measured concurrently and then subtracted from the observed enzymatic hydrolysis rates. Samples were stored at $0^{o}C$ until examined for alkaline phosphatase activity.

e) Succinate Dehydrogenase

Succinate dehydrogenase levels were determined by a modification of the spectrometric procedure described by Earl & Korner in 1965. In this assay system, 2,6-dichlorophenol-indophenol (DCIP) is reduced while sodium succinate is oxidized. The reduction of DCIP results in a decrease in absorbance at 600 mM.

The assay medium contained 50 mM potassium phosphate, pH 7.6, 1 mM KCN, 0.04 mM 2,6-dichlorophenol-indophenol, 20 mM sodium succinate, and 250 mM sucrose. The reaction was initiated by addition of membrane enzyme samples and observed for 10 minutes at $37^{o}C$ using a Gilford Model 2400 spectrophotometer. A standard curve was obtained each time to determine the amount of reduced 2,6-dichlorophenol-indophenol. Enzyme samples were stored at $-20^{o}C$ and then used immediately upon thawing.

f) Rotenone-Insensitive NADH Cytochrome c Reductase

Rotenone-insensitive NADH cytochrome c reductase was determined

by the methods of Matlib et al. (1979) and Sottocasa et al. (1967). The reduction of cytochrome c at 37°C was monitored at 550 nm on a Beckman Model 222 spectrophotometer. The assay medium contained 100 mM KH_2PO_4, pH 7.5, 250 mM sucrose, 5 mM KCN, 10 µM rotenone, 100 µM cytochrome c, and 150 µM NADH. NADH was added just prior to initiation of the reaction by addition of enzyme sample.

The concentration of reduced cytochrome c was calculated using the molar extinction coefficient for horse cytochrome c of $2.95 \times 10^{+4}$ M^{-1} cm^{-1}. Enzymatic rates were corrected for nonenzymatic reduction.

Samples were assayed for activity immediately after the isolation proceedure since rotenone-insensitive NADH cytochrome c reductase is quite labile and its activity is destroyed by freezing. Rotenone was dissolved in acetone and then added to the incubation medium (3 µl in 3.00 mls).

g) Monoamine Oxidase

Monoamine oxidase (MAO) was measured using the microfluorometric method of Krajl (1965) as modified by Century and Rupp in 1968. Kynuramine is degraded by monoamine oxidase to 4-hydroxyquinoline which can then be measured fluorometrically.

The reaction medium contained 50 mM $KHPO_4$ buffer, pH 7.4, and 0.1 mM kynuramine. The enzyme sample was added to give a final volume of 3 ml and the mixture was incubated at 37°C for 15 minutes. 2 mls of 0.6 M perchloric acid were added to the incubated mixture and the temperature reduced to stop the reaction. 1.00 ml of this suspension was mixed with 2.00 ml of 1N NaOH and the fluorescence intensity of each

sample was determined at 380 nm with an excitation wavelength of 315 nm. 4-Hydroquinoline concentrations were calculated from a standard curve, performed concurrently, linear in the range 25 nM to 7 µM. Nonenzymatic degradation rates were subtracted from values obtained with enzyme samples. Enzyme samples were stored in liquid nitrogen and used immediately after thawing.

h) NADPH Cytochrome c Reductase

NADPH cytochrome c reductase levels were measured using the method described for rotenone-insensitive NADH cytochrome c reductase. To measure this particular enzyme, NADPH replaced NADH in the assay medium. Again, nonenzymatic reduction was subtracted from enzymatic rates. Samples were used immediately after isolation due to the labile nature of NADPH cytochrome c reductase and its inactivation by freezing.

2.2.3 Characterization by Electron Microscopy

All samples were characterized by electron microscopy. F1, F2, F3, F4 and F5 were mixed with fixative (3% gluteraldehyde in Millonig's buffer) and centrifuged in SW-41 rotor at 150,000 g_{ave} for 13 hours. 1,000 g and 9,000 g pellets were fixed overnight in the regrigerator. All samples were postfixed for one hour in 1% osmium tetraoxide (diluted with 1 to 1 Millonig's buffer:water). The samples were washed three times with water and then dehydrated with graded ethanol concentrations (50% ethanol for 15 minutes, 70% for 15 minutes, 95% for 15 minutes, absolute ethanol twice for 10 minutes each), and finally with propylene oxide (twice for 10 minutes each).

The samples were then incubated in a mixture of 50% propylene

oxide and 50% araldite for three to four hours. After this, the samples were placed in 100% araldite and left overnight. They were then cured at 70°C for 2 days. The samples were sectioned using a Sorvall MT2 ultramicrotome. The sections were doubled stained: 15 minutes with uranyl acetate and then five to eight minutes with lead citrates. The sections were then viewed in a JEM7A transmission electron microscope. Sample preparation and viewing was carried out by the Electron Mircoscope Service of the Department of Pharmacology.

2.2.4 Fluorescence Studies

Fluroescence studies were performed using a Perkin-Elmer MPF-2A spectrofluorometer. Cuvette cells were maintained at various temperatures by a Haäke circulating water bath. Two probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 12-(9-anthroyl) stearic acid (12-AS), were employed. DPH was initially dissolved in tetrahydrofuran (THF) while 12-AS was dissolved in methanol. Final concentrations of DPH and 12-AS were 1×10^{-6} M and 2×10^{-6} M respectively. Membrane samples were diluted with 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 250 mM sucrose to yield a concentration of approximately 10 - 15 µg protein/ml.

a) Uptake

The fluorescent probe (either DPH or 12-AS) was added to the diluted membrane suspension and incubated at 44°C until an equilibrium had been reached. Equilibration was tested by observing the fluorescence intensity of the sample as a function of time. Once there was no further increase in fluoresence intensity, the membrane-probe system was said to be at equilibrium. The time taken to reach equilibrium was then used as

the incubation time for subsequent experiments.

b) Fluorescence Spectrum

Both excitation and emission spectra were obtained to determine characteristic spectra as well as excitation maxima and their corresponding emission maxima. The effect of temperature on both the overall shape of the curves and the excitation and emission maxima were examined at two temperatures, 44°C and 10°C. The effect of drugs on the spectra were also determined. This was performed with both fluorescent probes.

c) Fluoresence Polarization

Fluoresence polarization of both DPH and 12-AS calculated by examining the fluoresence intensities of the light, after passing through the sample, parallel to (I_{11}) and perpendicular to (I_1) the plane of polarization of the excitation beam. The degree of polarization, p, is defined by Steinberg (1975) as:

$$p = \frac{I_{11} - I_1}{I_{11} + I_1}$$

A correction factor is necessary to account for the unequal intensities of light dispersed by the emission monochromator of the spectrofluorometer into vertical and horizontal components of polarized light. This unequal transmission is strongly wavelenth dependent and thus must be accounted for at each emission/excitation setting. The corrected degree of polarization, p, is calculated using the following formula (Perkin-Elmer, 1969):

$$p = \frac{V_V - L_V \frac{V_H}{L_H}}{V_V + L_V \frac{V_H}{L_H}}$$

where the subscript indicates the direction of polarization of the light incident on the sample: for example L_V denotes the vertical component of incident light.

The "normo" letters indicates the direction of polarization of the light after it has passed through the sample. For example, L_V denotes the intensity of light in the vertical direction after passage through the sample where the plane of polarization of the incident light was in the horizontal direction (figure 2.2 for clarification).

Fluoresence polarization values were obtained for both excitation maxima (358 nm and 378 nm) with an emission maxima of 429 nm for DPH. The values for 12-AS were calculated for the following three settings expressed as excitation maximum / emission maximum: 257 nm / 437 nm, 364 nm / 437 nm, and 383 nm / 437 nm. The degree of polarization was examined over the temperature range of 44°C to 10°C by lowering the temperature of the cuvette in 5°C stages. The effect of histaminergic and adrenergic drugs on this profile was examined. All measurements were carried out using the more analytical, ratio mode of the Perkin-Elmer spectrofluorometer.

2.2.5 Tissue Experimentation

Guinea pigs, each weighing 350 to 500 g, were killed by a blow to the head. The heart was excised and placed in oxygenated Krebs solution (Edinburgh staff, 1974). Strips of the right ventricle were placed in organ baths of 15 ml under a resting tension of 500 mg. The strips were stimulated at a frequency of 2.5 Hz and a voltage of 20 V using a Grass 5D9 stimulator. Each tissue strip was allowed to equilibrate for 1 hour

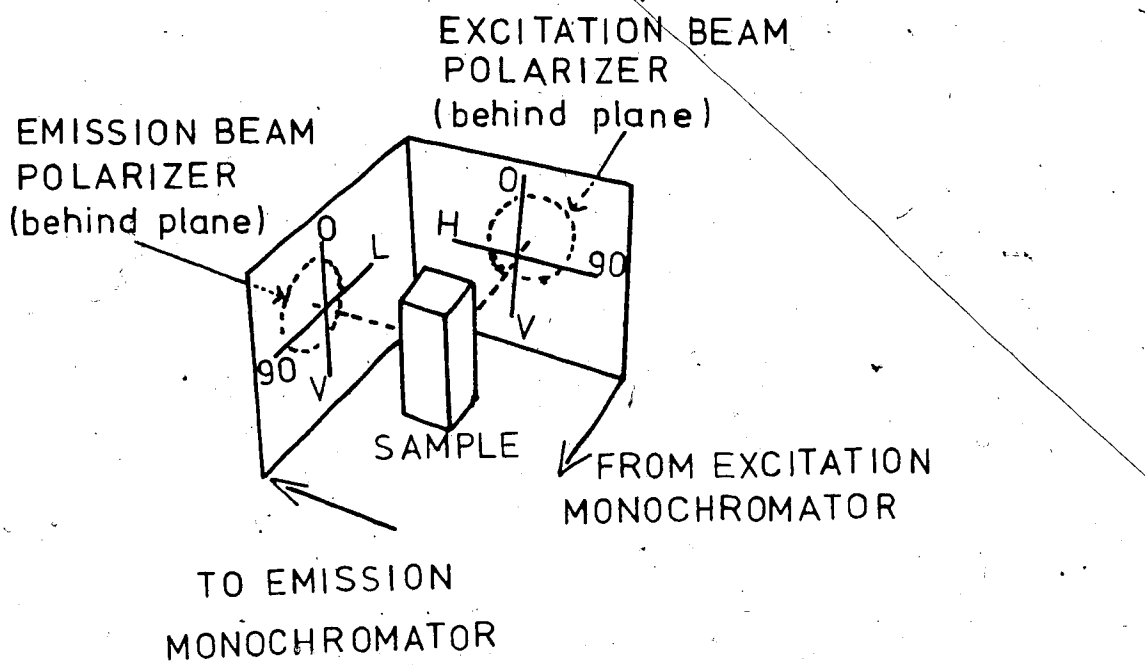


Figure 2.2. Representation of planes of incident and fluorescent light. Diagram obtained from Perkin-Elmer (1969).

at 37^{o} and the medium was changed every 30 minutes during this period.

Responses were recorded using a Grass FT03C strain gauge and Grass Model 7D polygraph. Cummulative dose response curves to both INA and histamine were performed. The effect of the probes and/or their corresponding solvents on the INA and histamine responses were examined.

CHAPTER 3

RESULTS

3.1 Purification and Characterization of Cardiac Sarcolemma Enriched with Histamine and β-Adrenergic Receptors

3.1.1 Purification of Histamine and β-Adrenergic Receptors

Purification of the cardiac sarcolemmal histamine H_2 and β-adrenergic receptors, as summarized in Figure 3.1, was achieved using standard membrane isolation techniques. Tissue disruption was accomplished by low shear homogenization using a Polytron® homogenizer. Cellular membranes were also fragmented by this method. Filtration of the suspension through cheesecloth removed connective tissue and unbroken tissue fragments.

Purification was carried out using differential centrifugation. Initial low speed (1,000 x g) centrifugation of this homogenate pelleted unbroken cells, nuclei, and some free contractile apparatus while membrane fragments and organelles were left in the supernatant. In order to increase the yield of the cardiac membrance fraction, the pellet was subjected to rehomogenization as well as refiltration and recentrifugation. The two supernatants were pooled. The pellet obtained at this stage contained intact cells and other subcellular debris such as intact mitochondria, some contractile apparatus, and a few membrane fragments as examined by electron microscopy (Figure 3.2). Further centrifugation (at 9,000 g_{max}) of the pooled supernatants removed most of the intact mitochondria as well as some contractile apparatus (Figure 3.3). The resultant supernatant, often referred to as the microsomal fraction, was then centrifuged at 110,000 g_{ave} for one hour. Further purification was then attempted using continuous sucrose gradient centrifugation. The

Figure 3.1. Schematic representation of the isolation proceedure for cardiac sarcolemma containing histamine and β-adrenergic receptors. See Materials and Methods section for further details.

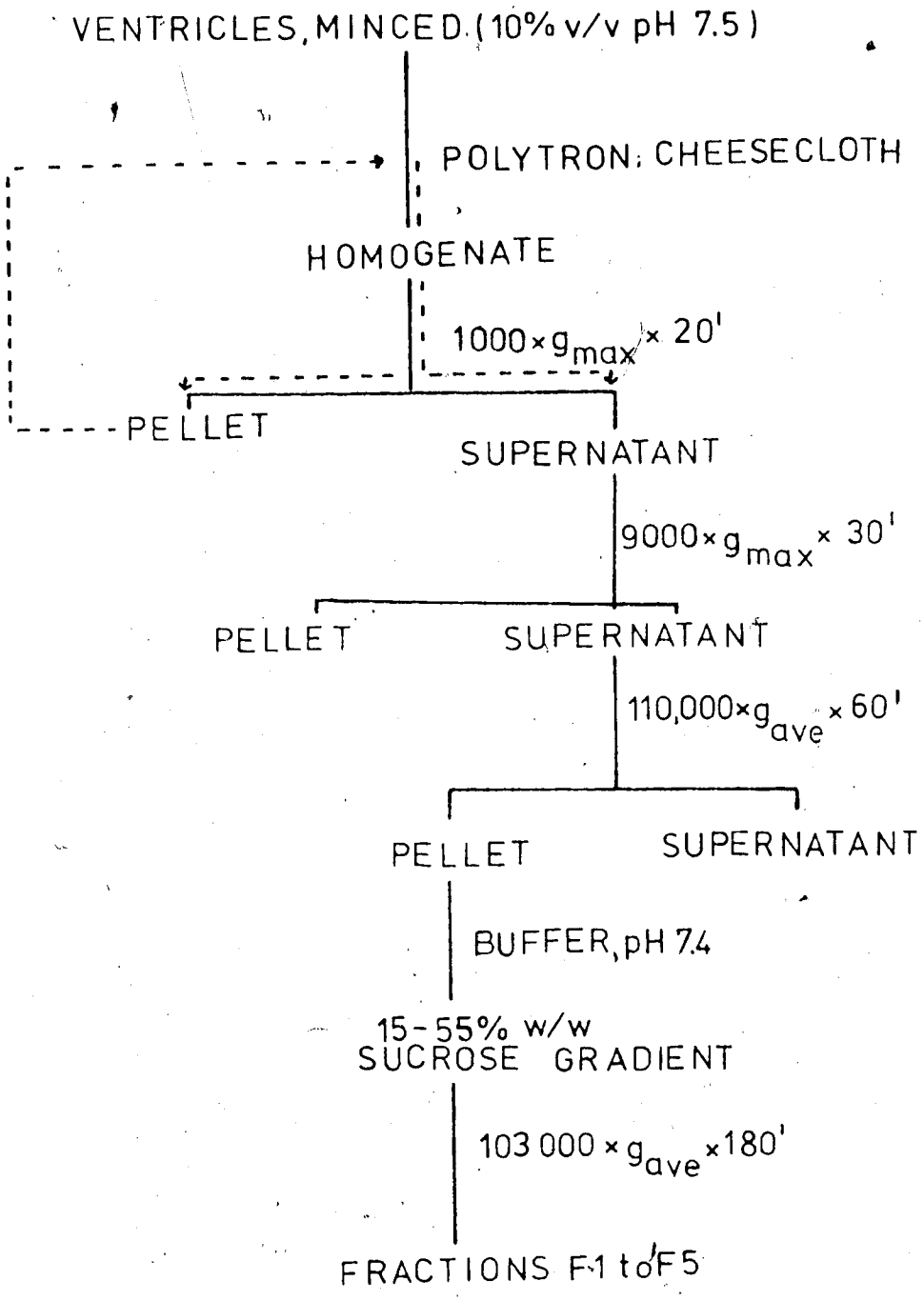
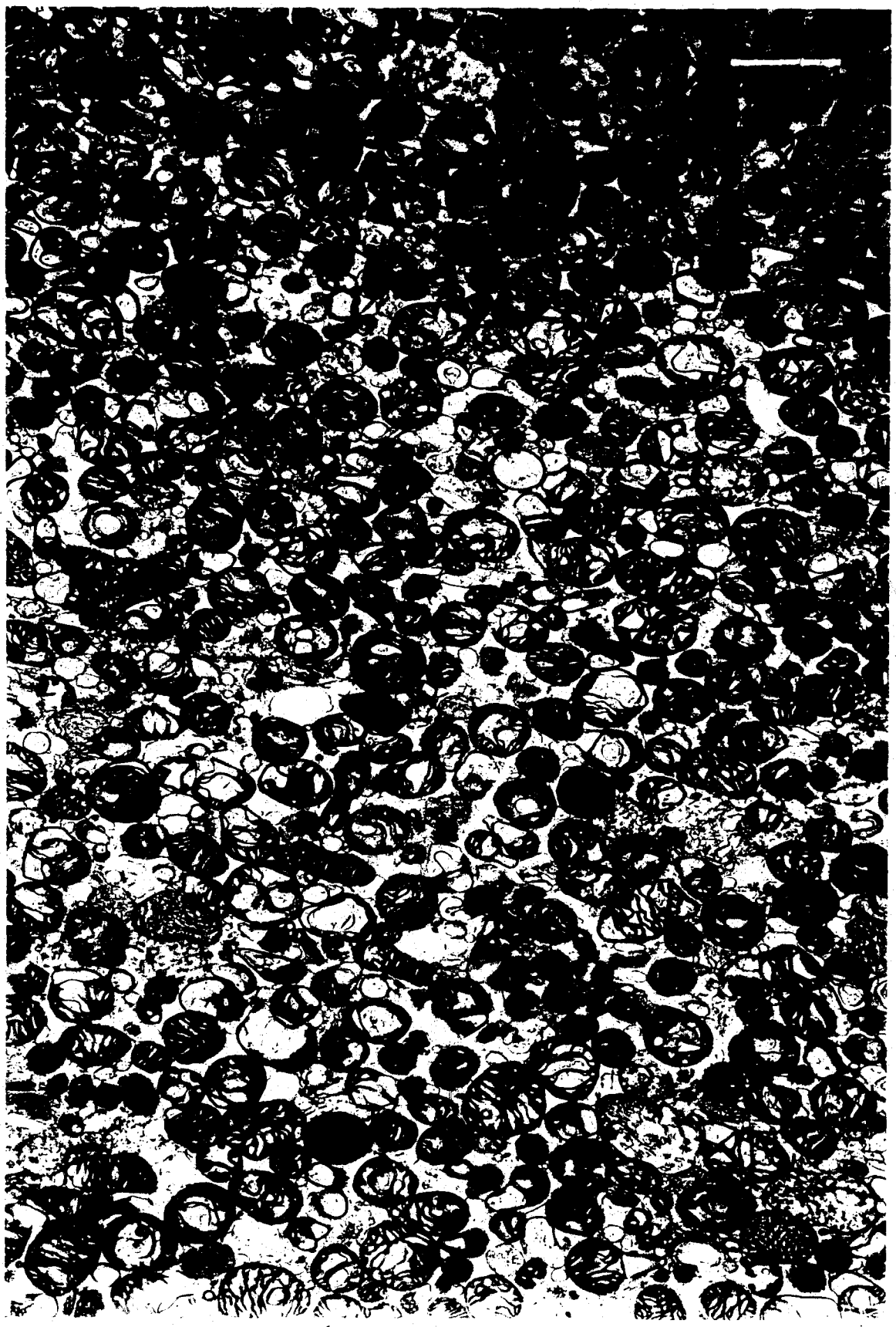

VENTRICLES, MINCED (10% v/v pH 7.5)
POLYTRON; CHEESECLOTH
HOMOGENATE
1000×g max × 20'
PELLET
SUPERNATANT
9000×g max × 30'
PELLET
SUPERNATANT
110,000×g ave × 60'
PELLET
SUPERNATANT
BUFFER, pH 7.4
15-55% w/w
SUCROSE GRADIENT
103 000 × g ave × 180'
FRACTIONS F1 to F5

Figure 3.2. Electron micrograph of 1,000 g_{max} pellet. The staining proceedure is described in Materials and Methods. The bar indicates 1 micron.

Figure 3.3. Electron micrograph of the 9,000 g_{max} pellet. The staining proceedure is described in Materials and Methods. The bar indicates 1 micron.



110,000 g pellet was resuspended in a minimal amount of buffer, loaded onto the gradient, and centrifuged at 103,000 g_{ave} for three hours. After this time, five bands were obtained (Figure 3.4). The fractions occurred at 12%, 14%, 27%, 34% and 40% w/w sucrose and labelled F1 through F5, respectively. F1 and F2 were distinguished by their difference in colour. F2 appeared to have an orange hue. F3 and F4 were arbitrarily divided (see Discussion), with F4 being more opaque than F3. F5 was always appeared as a very sharp band at 40% w/w sucrose. The sharpness suggests that this fraction may be homogeneous with respect to sedimentation properties.

The protein distribution is presented in Table 3.1. The percentage of protein in each gradient fraction ranged from about 0.06% for F1 to about 0.58% for F4. Most of the protein in the preparation was found in the 1,000 g pellet which contained unbroken cells, nuclei, contractile apparatus, and some free mitochondria. The amount in the gradient fractions could perhaps be increased by a third and possibly a fourth rehomogenization. It was felt that although the yield could increase, more damaged sarcolemma, its receptors and other proteins would result. The actual yield of protein was such that the 0.58% value for the F4 fraction represents approximately 2.5 to 3.5 mg.

The fractions F1 through F5 were then examined for the presence of histamine H_2 receptors and β-adrenergic receptors. Since agonist interaction with these two receptors is known to activate adenylate cyclase, the ability of histamine and isoproterenol (INA) to stimulate this enzyme was used to indicate the precense of these two receptors.

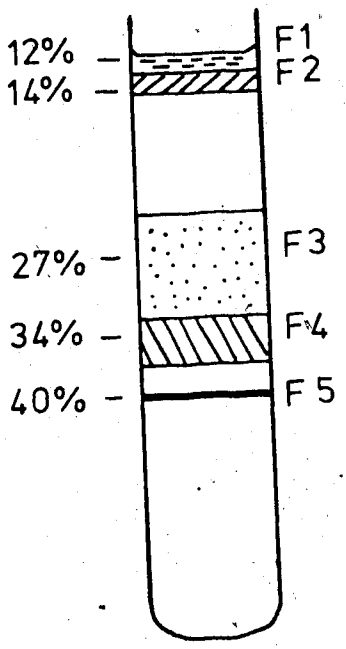


Figure 3.4. Positioning of the five fractions on the sucrose gradient. Numbers at the left indicate Mean position of each band, expressed as % w/w sucrose.

TABLE 3.1

Distribution of Protein Throughout the Various Fractions

Fraction	% Total Protein
F1	0.06 ± 0.02 [a]
F2	0.10 ± 0.01
F3	0.28 ± 0.06
F4	0.58 ± 0.09
F5	0.07 ± 0.02
1,000 g pellet	78.8 ± 1.3
9,000 g pellet	4.8 ± 1.0
Supernatant	15.4 ± 1.2

[a] expressed as Mean ± S.E.M. (n = 8)

The purification of adenylate cyclase was first examined. From Figure 3.5, it can be seen that adenylate cyclase activity could not be detected at all in fractions F1 and F2. Even if the incubation time was doubled no activity was observed. On the other hand, both fractions F3 and F4 were purified over ten fold with respect to adenylate cyclase. The adenylate cyclase in the F5 fraction was enriched about six fold.

Sodium fluoride stimulation of adenylate cyclase was then tested as a control measure to ensure that the enzyme was capable of undergoing activation (Figure 3.6). NaF produced an increase in activity in the range 120 to 190% in the three fractions, F3, F4 and F5.

Histamine and INA stimulation of the adenylate cyclase in those three fractions were then tested in the presence of 10^{-5} M GTP. The GTP was required for maximal activation. In both the F3 and the F5 fractions, an increase of about 75% ($\pm$ 44% and $\pm$ 23%, respectively) was found when histamine was employed while in F4, the enhancement of activity was about 35 $\pm$ 14%. Thus these results suggest the presence of functional histamine receptors in F3, F4 and F5.

INA stimulation of adenylate cyclase produced a 30 $\pm$ 2% enhancement of the basal activity in fraction F3. INA appeared to have no effect on activity in fraction F4 while in F5, the presence of INA had variable effects - on some occasions, causing adenylate cyclase stimulation and on others, not activating the enzyme. Thus, the F3 fraction appears to contain functional β-adrenergic receptors while the other two fractions do not consistently contain them.

In summary, histamine receptors were demonstrated in F3, F4 and F5

Figure 3.5. Histogram of adenylate cyclase distribution in the five sucrose gradient fractions. RSA, or relative specific activity, is the specific activity of the particular fraction divided by the total specific activity of the preparation, where total specific activity is the total activity divided by the total protein. The width of the histogram reflects the amount of protein in each fraction. Adenylate cyclase was not detected in either F1 or F2; hence, the relative amount of protein is not shown for these two fractions. Results are expressed as Mean $\pm$ S.E.M. of 4 preparations.

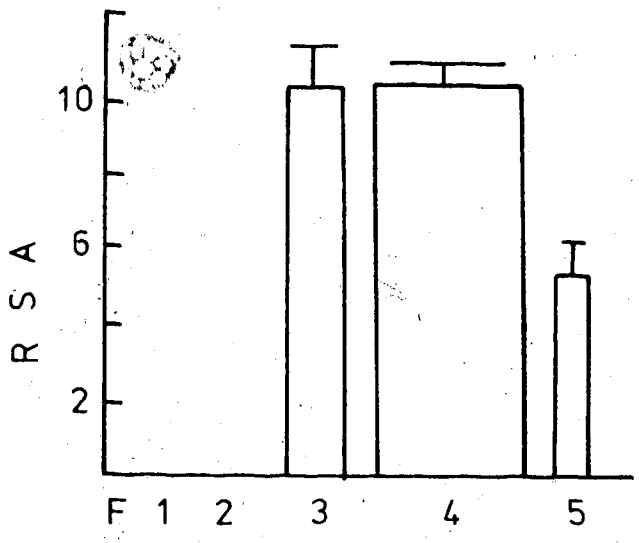
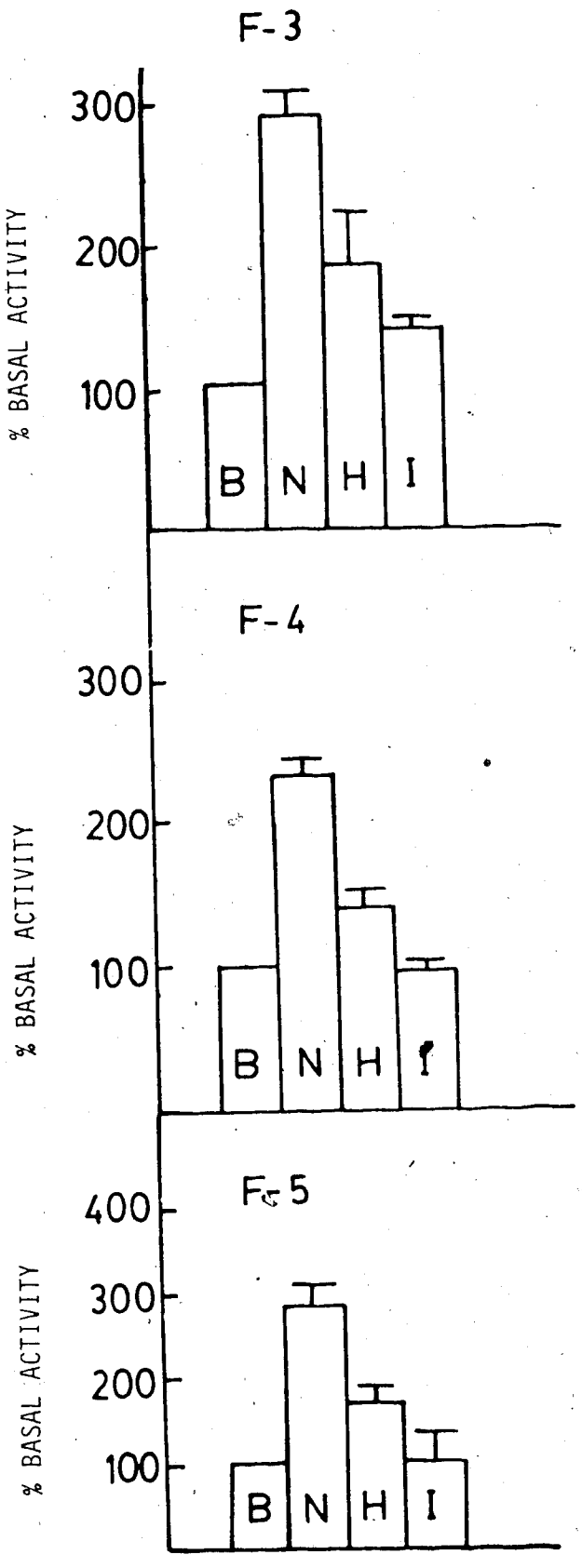

RSA
10
6
2
F 1 2 3 4 5

Figure 3.6 Histograms showing stimulation of adenylate cyclase in fractions F3, F4, and F5 by N = NaF (10^{-2} M), H = histamine (10^{-4} M), and I = INA (10^{-5} M) in comparison to B - basal activity. Both H and I were examined in the presence of GTP (10^{-5} M). GTP at this concentration did not appear to affect basal activity. Basal activity normalized to 100%. N, H, and I values expressed as % of basal activity. Results are expressed as Mean $\pm$ S.E.M. for 4 preparations.


F-3
300
200
100
% BASAL ACTIVITY
B
N
H
I
F-4
300
200
100
% BASAL ACTIVITY
B
N
H
I
F-5
400
300
200
100
% BASAL ACTIVITY
B
N
H
I

while only F3 showed any consistent presence of β-receptors.

3.1.2 Enzymatic Characterization

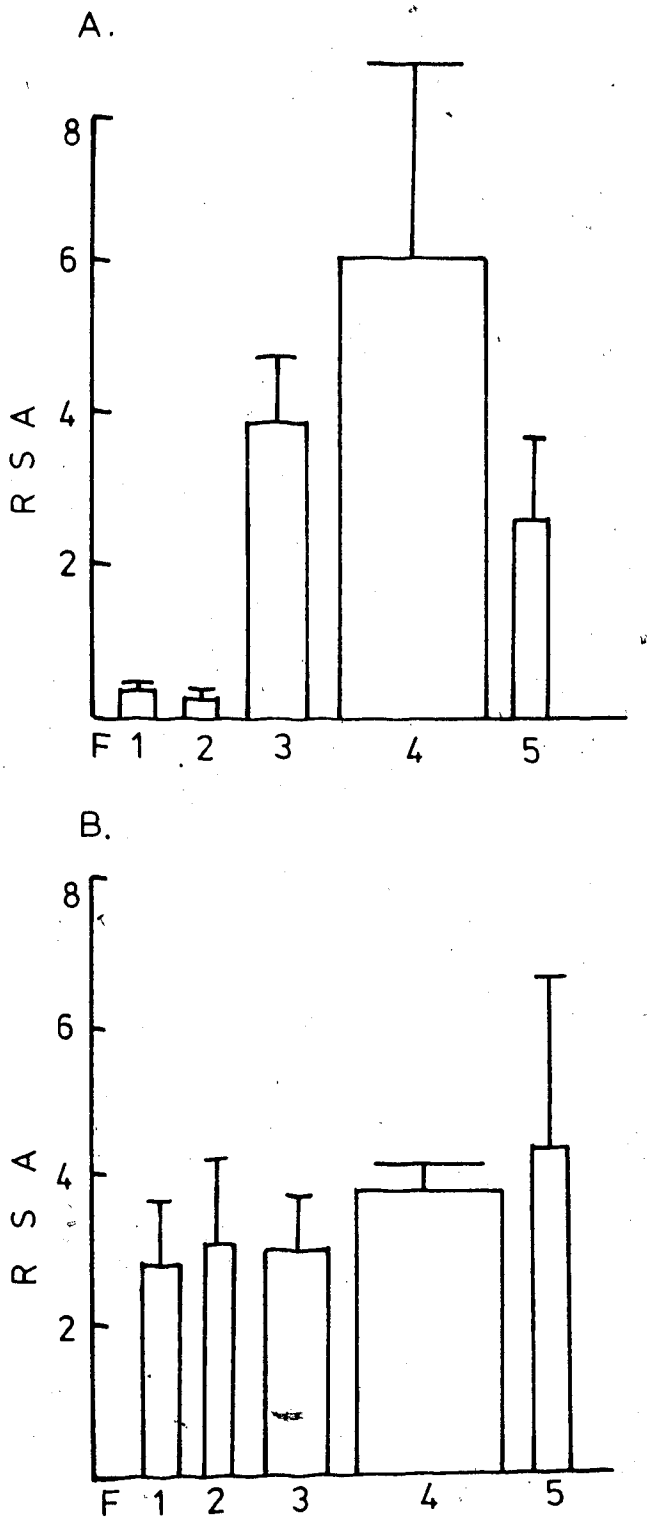
Further characterization was then performed on the five sucrose gradient fractions. Two other sarcolemma markers were employed (Figure 3.7). When $(Na^+ + K^+)$-ATPase was examined as to its distribution, fractions F1 and F2 were found to contain little of this enzyme. Purification occurred in fractions F3, F4 and F5 with the greatest, but most variable envichment occurring in fraction F4. The profile of the other enzyme, alkaline phosphatase, is shown in Figure 3.7B. Purification of this enzyme marker occurred in all five fractions with little difference between fractions.

Characterization of these samples also include examination of possible contamination. The presence of inner mitochondrial membranes was examined by using the enzyme marker, succinate dehydrogenase (Figure 3.8). The order of contamination of this enzyme was fractions F1 and F2, fractions F3 and F5 and fraction F4. However, the degree of contamination of any of these fractions was less than the homogenate. In particular fractions F1 and F2 showed negligible contamination.

The presence of the outer mitochondrial membrane, which is easily removed from the mitochondrion during isolation proceedures, was determined by two enzyme markers, rotenone insensitive NADH cytochrome c reductase and monoamine oxidase (MAO) (Figure 3.9). F1 had reduced contamination of the first enzyme while F2 had somewhat more. F3 showed contamination with an increasing amount in F4 and F5. F1 and F2 contained negligible MAO while F3 through F5 showed contamination by this enzyme.

NADPH cytochrome c reductase was employed as a measure of possible

Figure 3.7. Histograms showing the RSA with respect to two putative plasma membrane markers, (A) ($Na^+ + K^+$)-ATPase and (B) alkaline phosphatase, in the five sucrose gradient fractions. The width of the histogram reflects the amount of protein in each fraction. Results are expressed as Mean $\pm$ S.E.M. of 4 preparations.


A.
8
6
4
2
R S A
F 1 2 3 4 5
B.
8
6
4
2
R S A
F 1 2 3 4 5

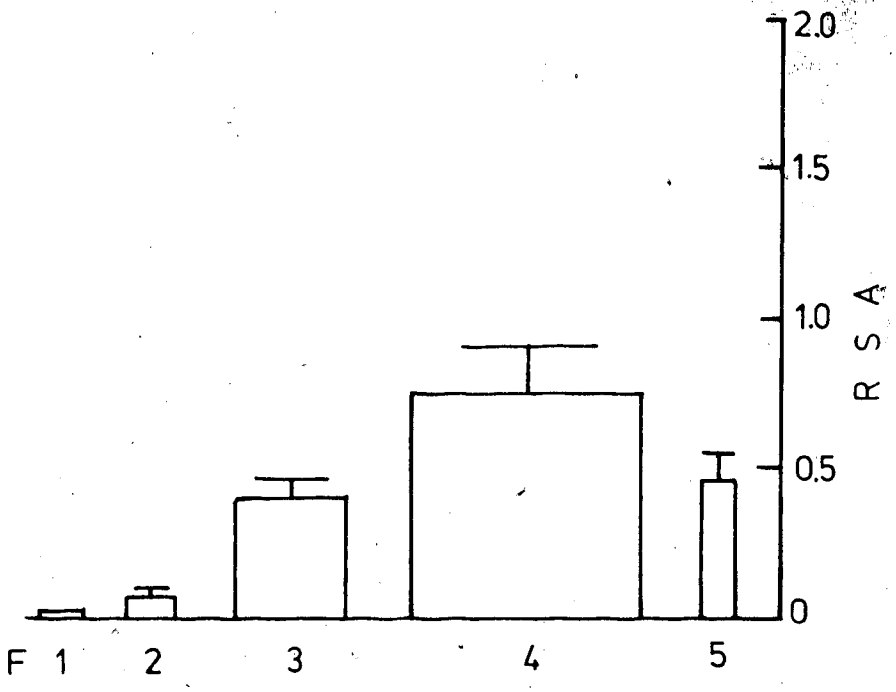
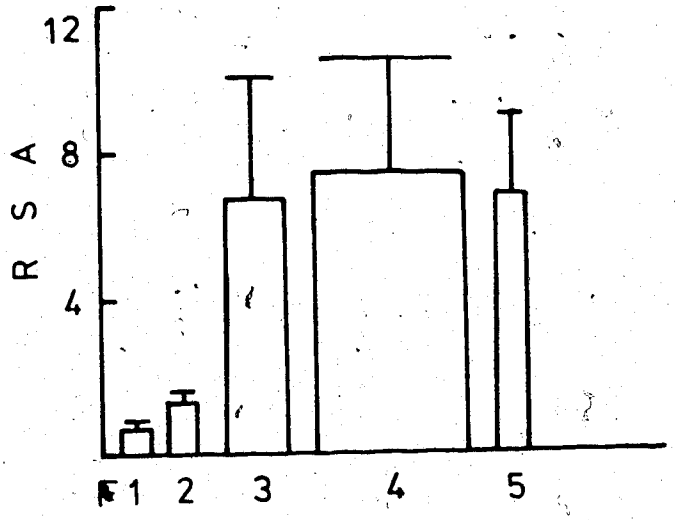


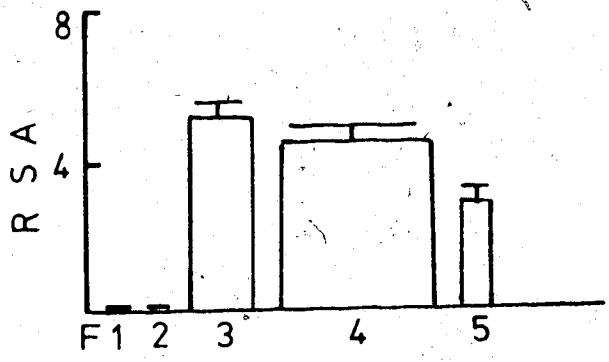
Figure 3.8. Histogram showing the RSA with respect to succinate dehydrogenase in the five sucrose gradient fractions. The width of the histogram reflects the amount of protein in each fraction. Results are expressed as Mean ± S.E.M. of 4 preparations.

Figure 3.9. Histograms showing the RSA with respect to (A) rotenone-insensitive NADH cytochrome c reductase and (B) monoamine oxidase in the five fractions. The width of the histogram reflects the amount of protein in each fraction. Results are expressed as Mean ± S.E.M. of 4 preparations.

A.



B.



sarcoplasmic reticulum contamination. As can be seen from Figure 3.10, all fractions contained this enzyme marker with the extent of contamination increasing from F1 through to F5.

3.1.3 Characterization by Electron Microscopy

After enzymatic characterization, the gradient fractions were stained for protein and examined by electron microscopy. F1 and F2 were found to consist of particulate protein (Figures 3.11 and 3.12). A few vesicles and intact mitochondria were also observed but they were insignificant when compared with the amount of particulate protein present. Thus these two fractions can be said to contain little membrane bound protein.

The electron micrographs of F3 showed vesicles with an average diameter of approximately 110 nm (a range of 78 to 200 nm). An example is shown in Figure 3.13. It appears that there were no intact mitochondria. Fraction F4 (Figure 3.14) was also vesicular in nature in the range of 65 to 375 nm. A few intact mitochondria and some particulate protein could be observed. Fraction F5 (Figure 3.15) was found to contain membrane vesicles, ranging in size from 50 to 310 nm. A few intact mitochondria and some more dense vesicles could also be observed.

Sedimentation of fractions F1 and F2 presented a great problem. Fixative was added prior to centrifugation and the centrifugation time was extended to 13 hours. F1 and F2 pellets could now be observed after osmic acid staining. Thus the difficulty in pelleting these two fractions suggests that the protein in these fractions is of the soluble, nonaggregated form.

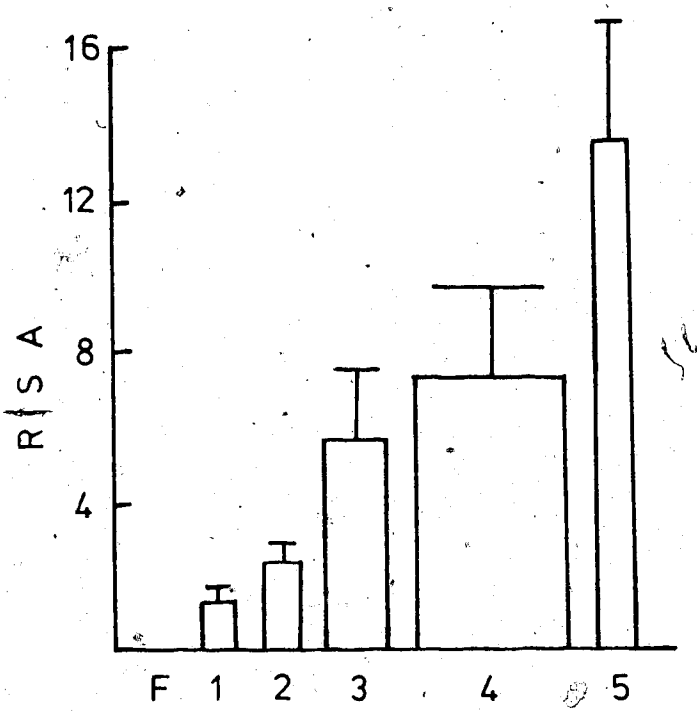
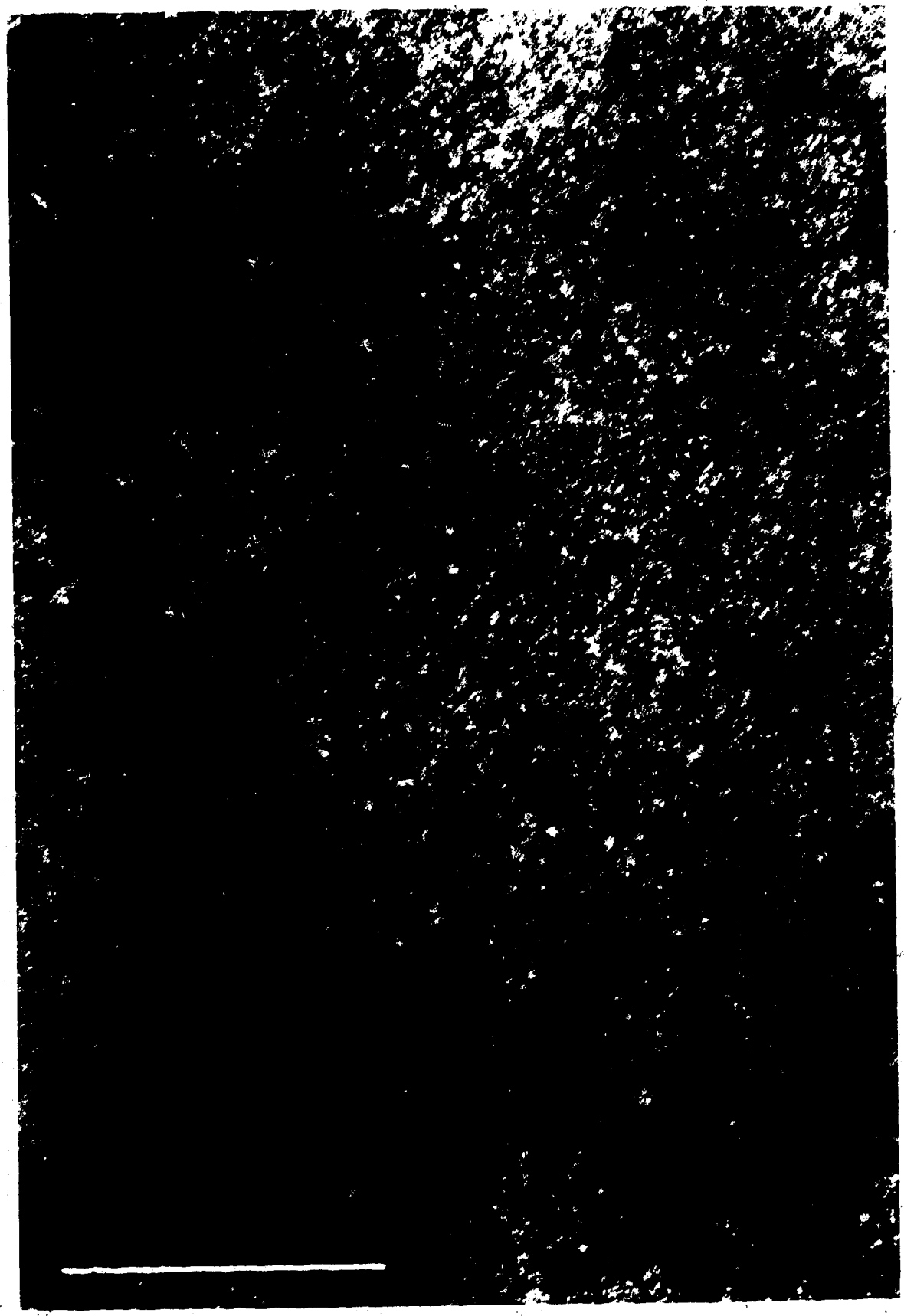


Figure 3.10. Histogram showing the RSA with respect to NADPH cytochrome c reductase in the five sucrose gradient fractions. The width of the histogram reflects the amount of protein in each fraction. Results are expressed as Mean ± S.E.M. of 4 preparations.

Figure 3.11. Electron micrograph of sucrose gradient fraction F1. Preparation and staining is described in Materials and Methods. The bar indicates 1 micron.



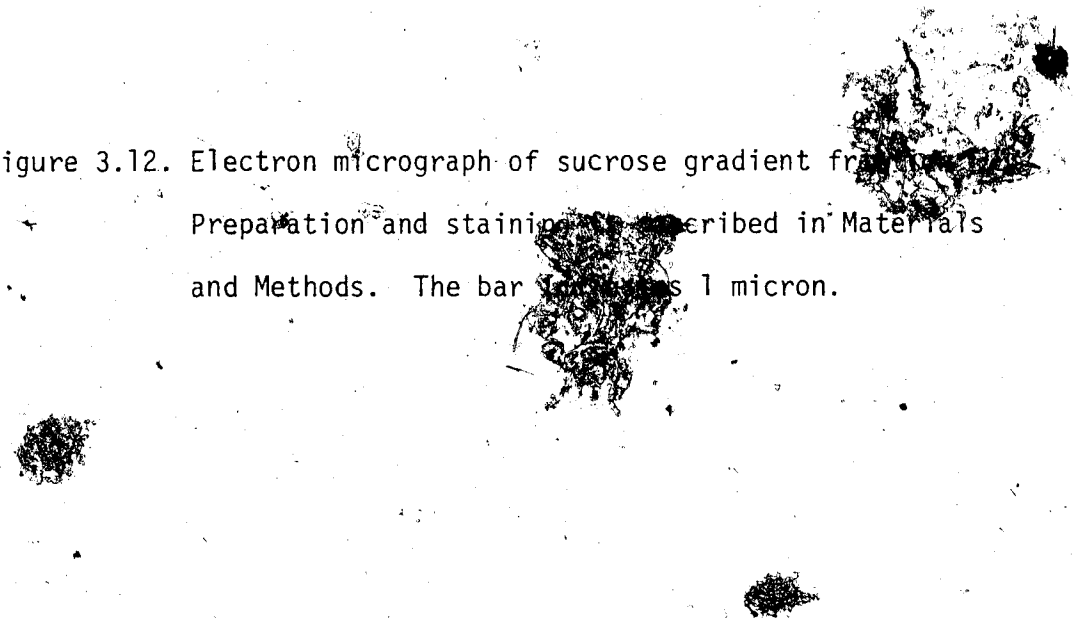


Figure 3.12. Electron micrograph of sucrose gradient fr[illegible]
Preparation and staini[illegible]scribed in Materials
and Methods. The bar [illegible]s 1 micron.

Figure 3.13. Electron micrograph of sucrose gradient fraction F3. Preparation and staining is described in Materials and Methods. The bar indicates 1 micron. The vesicular membrane fragments appear to range in size between 78 and 200 nm.

Figure 3.14. Electron micrograph of sucrose gradient fraction F4. Preparation and staining is described in Materials and Methods. The bar indicates 1 micron. The diameter of the vesicular fragments appears to range in size between 65 and 375 nm.

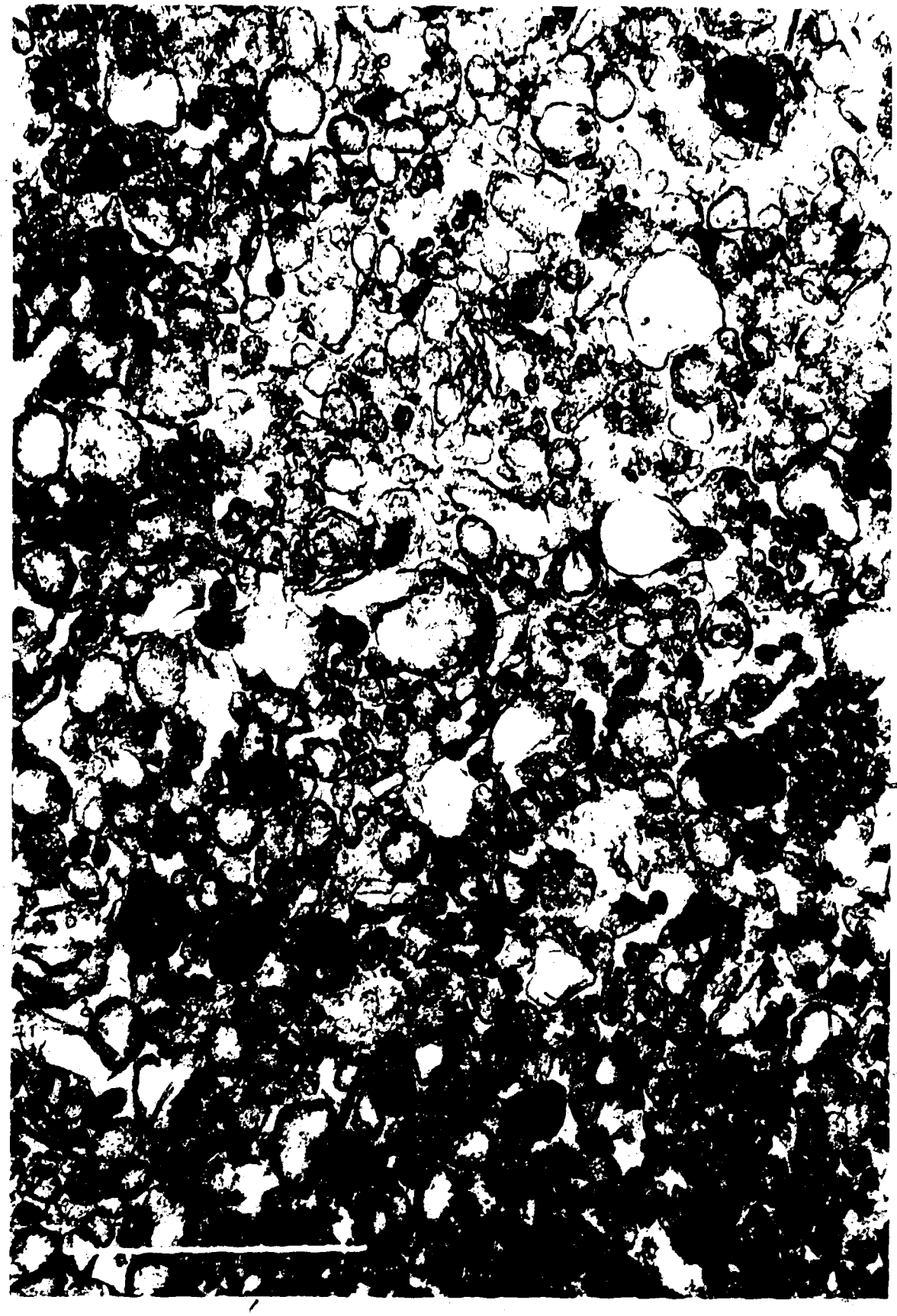


Figure 3.15. Electron micrograph of sucrose gradient fraction F5. Preparation and staining is described in Materials and Methods. The bar indicates 1 micron. The diameter of the vesicular fragments appears to range in size between 50 and 310 nm.

3.1.4 Summary of Characterization Findings

The F3 fraction appears to be the fraction of choice. F3 was the one fraction found to contain both histamine and β-adrenergic receptors as examined by histamine and INA stimulation of adenylate cyclase. Furthermore, two commonly employed plasma membrane markers, $(Na^+ + K^+)$-ATPase and alkaline phosphatase, were enriched in this fraction. Contamination of the inner mitochondrial membrane was reduced but there did appear to be some contamination of the outer mitochondrial membrane and sarcoplasmic reticulum. Electron microscopy revealed unilamellar vesicles of approximately 110 nm diameter.

Thus the F3 fraction was employed in further studies of drug-receptor interactions, their effects on the lipid bilayer, and the possibility of thermal interconversion of one receptor subtype to another using the biophysical technique of flourescence spectroscopy.

3.2 Fluorescence Studies

The concentration of the two probes and the F3 fraction were decided by examining the literature for initial values (Akiyama and Igisu, 1979; Charnock and Bashford, 1975; Elferink, 1977; Luly and Shinitzky, 1979; Mély-Goubert and Freedman, 1980; Rooney et al., 1979; Spero, 1978; Waggoner and Stryer, 1970; Wallach et al., 1970; Zierler and Rogus, 1978) and then adjusting the concentrations to the yield of the F3 fraction and to sensitivity of the fluorescence spectrophotometer.

3.2.1 Uptake

Equilibration of each lipophilic probe with the membrane fraction F3 was examined at the highest temperature employed, 44°C. Equilibrium at this temperature is attained more rapidly than at the lowest temperature, 10°C. For the probe 1,6-diphenyl-1,3,5-hexatriene (DPH), the maximum fluorescence intensity was reached within four to eight minutes of addition of DPH (Figure 3.16). The intensity then decreased slightly and stabilized within about 40 minutes. This 40 minute time figure was then used as the minimum incubation time for further studies.

The uptake of 12-AS into the F3 vesicle was also observed (Figure 3.17). The equilibrium fluorescence intensity maximum was obtained after a longer incubation time of about 20 minutes. Once the maximum was reached the fluorescence remained constant from then on. The minimum incubation time used was thus 25 minutes.

3.2.2 Fluorescence Spectra

For each experiment, the excitation and emission spectra were

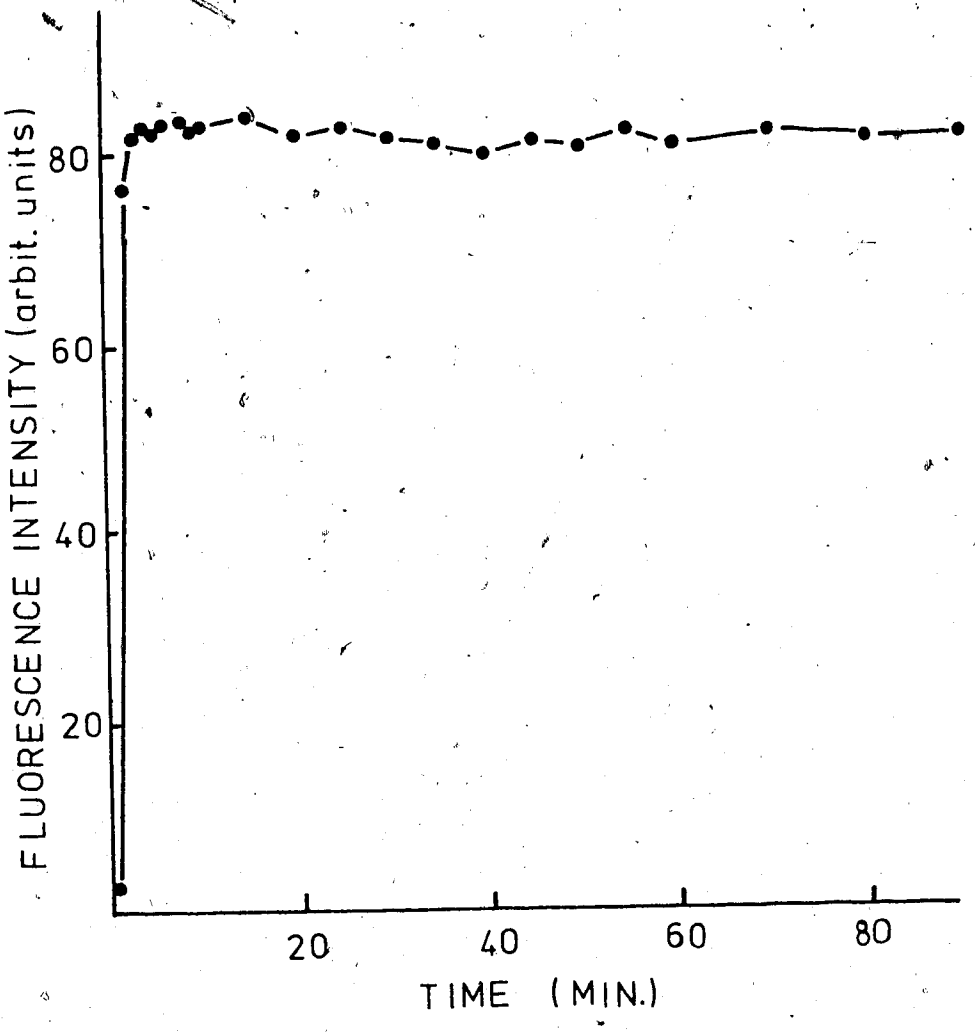


Figure 3.16. Flourescence intensity of DPH (10^{-6} M) as a function of incubation time. Membrane protein concentration was 12 µg/ml. Ex. = 358 nm; Em. = 429 nm.

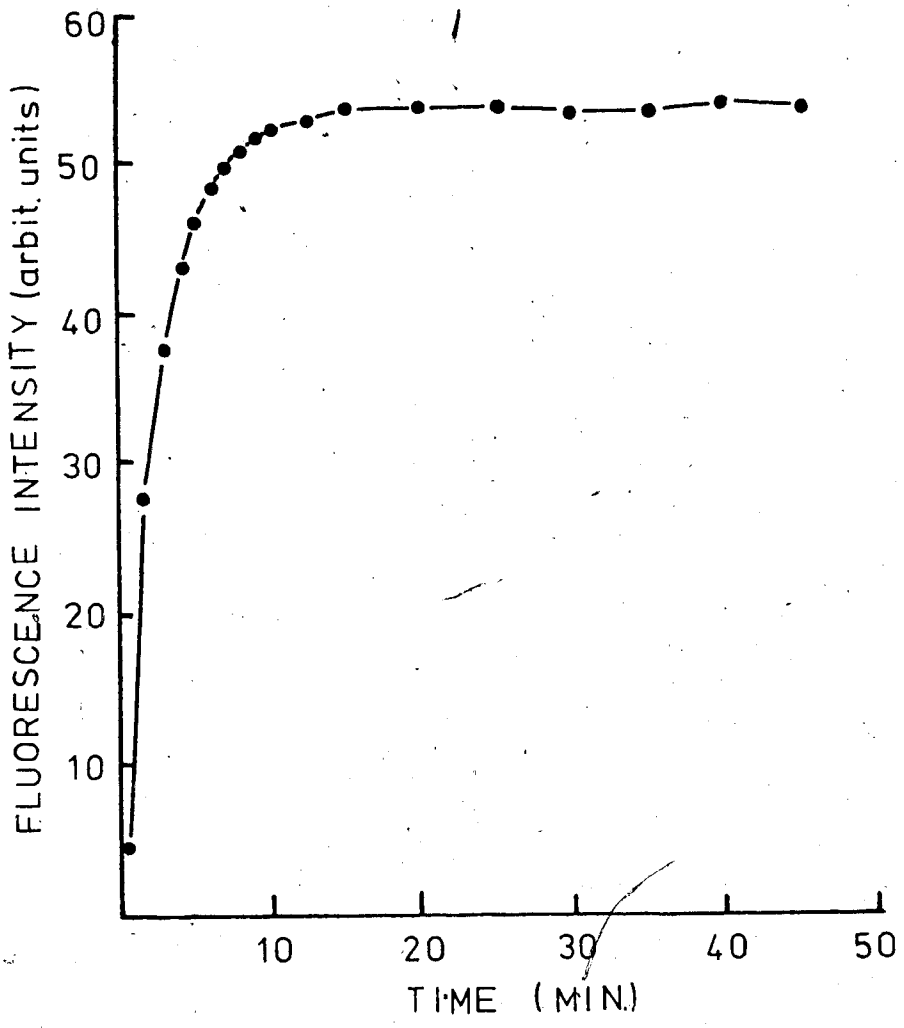


Figure 3.17. Fluorescence intensity of 12-AS (2×10^{-6} M) as a function of incubation time. Membrane protein concentration was 12 µg/ml. Ex. = 365 nm; Em. = 435 nm.

recorded in order to determine the wavelength values for the emission and excitation maxima to be used for fluorescence polarization. A representative sample for the lipophilic probe DPH is illustrated in Figure 3.18. For the excitation curve, the emitted light was observed at a wavelength of 430 nm. The excitation wavelength was then varied from 300 to 400 nm. Two peaks were observed: one at 358 $\pm$ 1 nm and the other at 378 $\pm$ 1 nm, with the former reaching a higher intensity of emitted light. In addition, a shoulder at about 340 nm was consistently present.

The emission spectrum of DPH in fraction F3 is shown in Figure 3.18B. The wavelength at which the maximum intensity (358 nm) was observed in the excitation spectrum was set as the excitation wavelength. The emission wavelength was then varied from 390 nm to 480 nm. An emission maximum was obtained at 428 $\pm$ 1 nm. A shoulder was consistently observed at about 408 nm and another at about 450 nm.

The lower curves in the figure are recorded from DPH in the buffer system in the absence of membrane. Thus the spectrum of the DPH/F3/buffer sample did not arise from the presence of the buffer or the solvent THF which was used to dissolve the probe.

The same proceedure was performed for the second probe, 12-AS (Figure 3.19). For the excitation spectrum, the emission wavelength was set at 435 nm and the excitation wavelength was varied from 230 to 400 nm. Three major peaks were observed: a solitary, sharp peak at 257 $\pm$ 1 nm, a second peak at 364 $\pm$ 1 nm, and a third peak at 383 $\pm$ 1 nm. A minor peak and a shoulder were present at about 348 nm and 332 nm, respectively.

Figure 3.18. Spectra of DPH (10^{-6} M) in fraction F3 and buffer at 44°C. (A) Excitation spectrum with emission observed at 430 nm. (B) Emission spectrum with sample excited at 358 nm. The corresponding blanks represent DPH in buffer only.

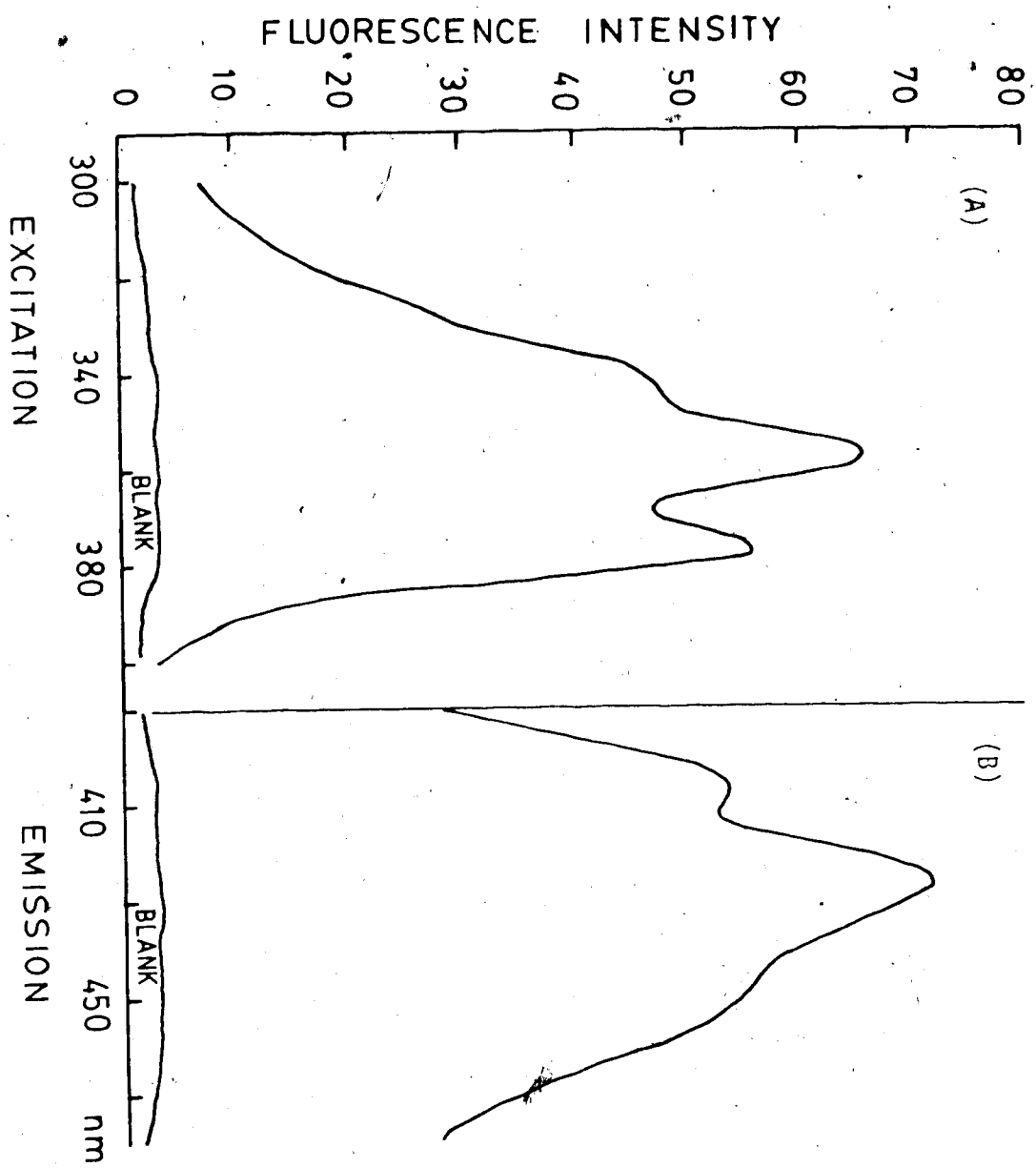
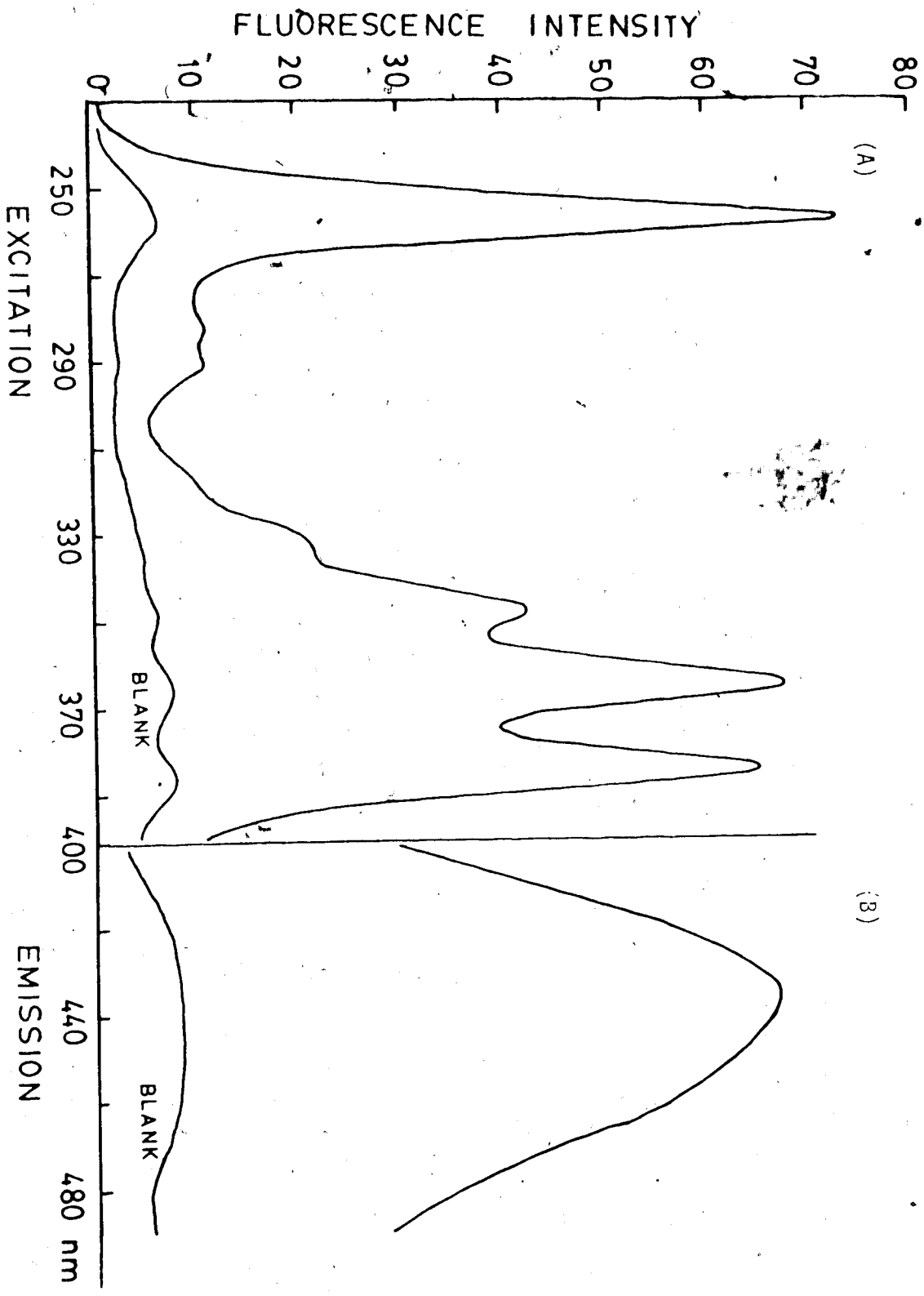

FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
80
(A)
(B)
EXCITATION
300
340
380
BLANK
EMISSION
410
450
nm
BLANK

Figure 3.19. Spectra of 12-AS (2×10^{-6} M) in F3/buffer at 44°C. (A) Excitation spectrum with emission observed at 435 nm. (B) Emission spectrum with sample excited at 364 nm. The corresponding blanks represent 12-AS in buffer only.

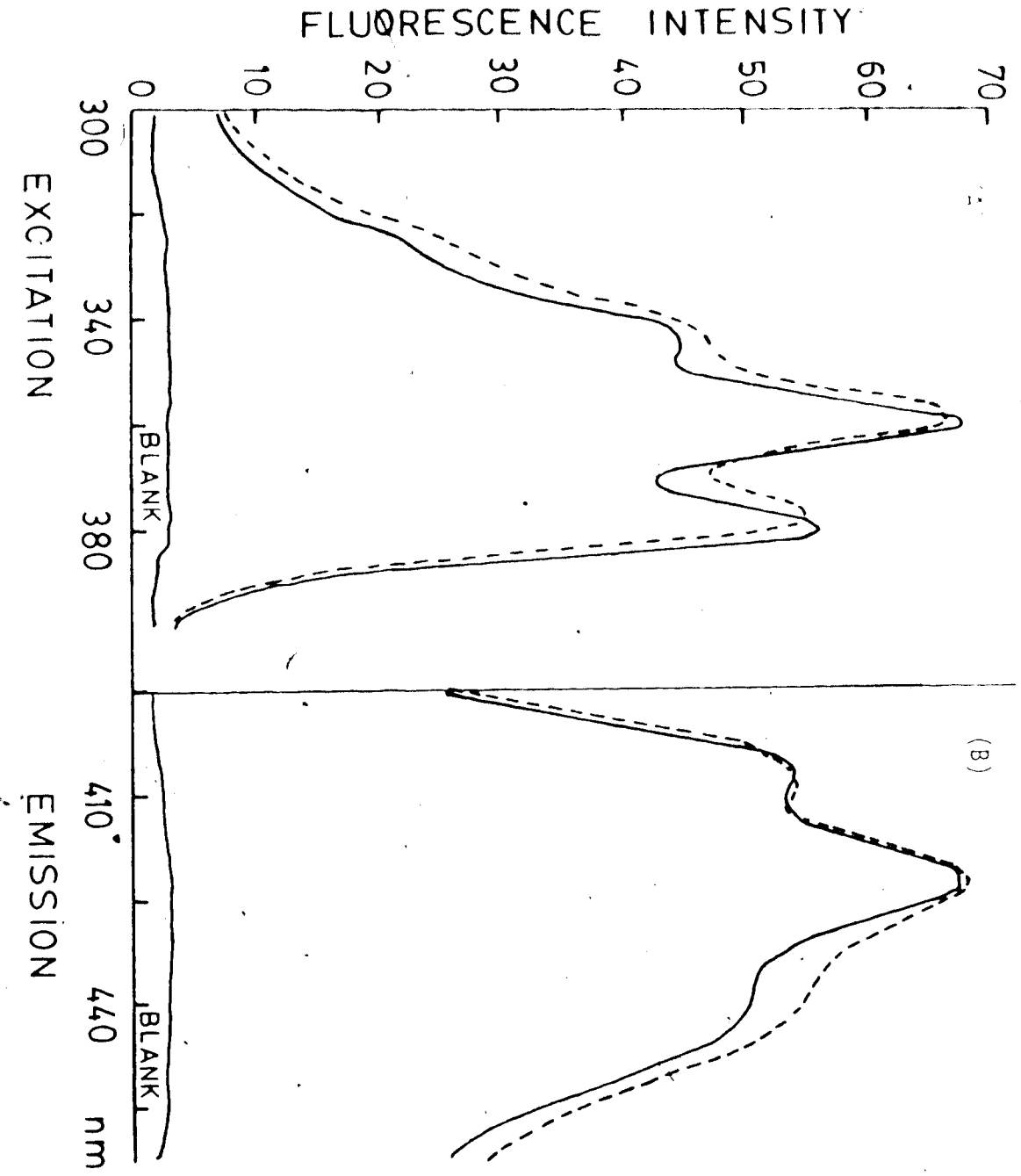

FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
80
(A)
250
290
330
370
400
440
480 nm
EXCITATION
EMISSION
BLANK
BLANK
(B)

The emission spectrum was then examined (Figure 3.19B). A broad peak with a maximum at about 437 nm was obtained. If an excitation wavelength of either 257 nm or 383 nm was employed, a similar emission spectrum was obtained. The only difference appeared to be the resultant intensity of emitted light.

Again the lower curves were obtained from the probe, 12-AS, in the buffer system. Here again, the excitation and emission spectra of the sample containing F3 resulted from the F3 membrane fraction instead of either the buffer system or methanol, the solvent used to dissolve 12-AS.

The influence of temperature on the spectra of DPH and 12-AS was examined. Figure 3.20 illustrates the DPH spectra at both 44°C and 10°C. Both excitation and emission spectra appear to be sharper. In examining the excitation spectrum, the decrease in temperature caused the 358 nm peak to shift about 2 nm to a longer wavelength (a red shift) as well as a red shift of about 3 nm in the 378 nm peak. The shoulder at 340 nm became more pronounced. The intensities of the 358 and 378 nm peaks was found to increase slightly with the decrease in temperature. The corresponding control did not change significantly from 44°C to 10°C. The emission spectrum at 10°C appeared to very similar to the one at 44°C. The shoulder at approximately 408 was essentially unchanged. The peak at 428 nm, which was not shifted at 10°C seemed to have increased somewhat in intensity. The major difference between the 10°C and 44°C spectra was found from about 435 nm to 480 nm. The intensity of emitted light was consistently reduced in this region at 10°C. Again the blank did

Figure 3.20. Temperature effect on the spectra of DPH (10^{-6} M) in F3/buffer. (A) Excitation spectra; Em. = 430 nm. (B) Emission spectra; Ex. = 358 nm. (----------) 44°C; (————————) 10°C. Spectra of blank (DPH plus only buffer) remained the same.


FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
300
340
380
410
440
nm
EXCITATION
EMISSION
BLANK
BLANK
(B)

not differ significantly at the two temperatures.

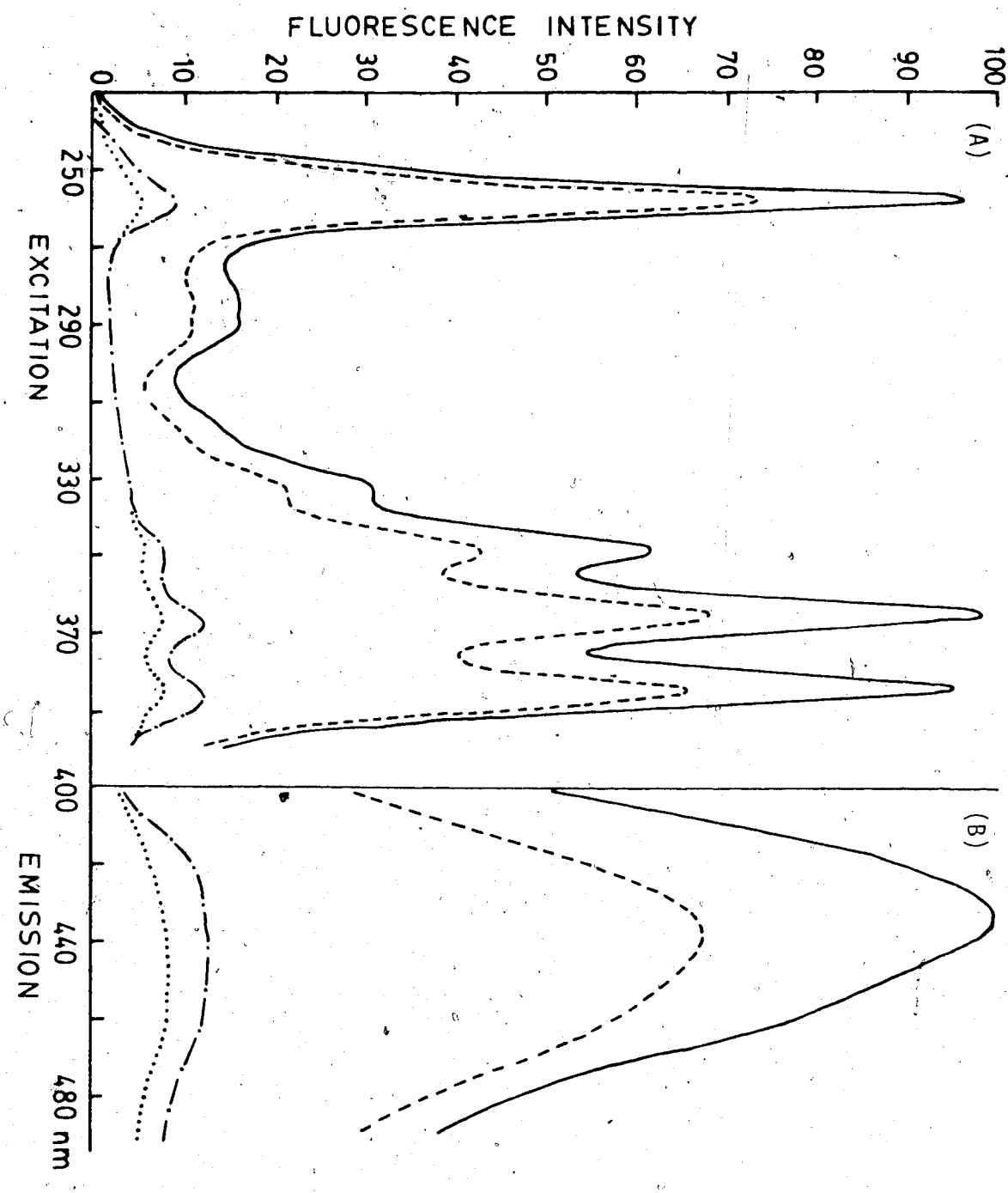
With 12-AS as the probe, the fluorescence intensity for both the excitation and emission spectra were increased substantially at 10°C (Figure 3.21). The corresponding controls showed a slight increase in their intensities at the lowered temperature. There appeared to be no change in the excitation maxima nor in the emission maximum with the reduction in temperature.

Having examined the temperature effects on the fluorescence spectra of DPH and 12-AS, the possibility of drug-induced alterations in the spectra was probed. Agents such as histamine, the two H_2 antagonists cimetidine and metiamide, the two H_1 antagonists chlorpheniramine and diphenhydramine, the adrenergic agonists INA, noradrenaline and phenylephrine, the β-adrenergic antagonist propranolol and the α-adrenergic antagonist phentolamine were employed. In all cases, and with both the probes, no wavelength shift nor intensity change was observed at either 44°C or 10°C (results not illustrated), and thus the spectra looked identical to those of samples containing just the probe, F3 fraction and buffer system. Thus no drug receptor interaction could be detected directly or indirectly by either wavelength shifts or intensity changes (either enhancement or quenching of the fluorescence).

3.2.3 Fluorescence Polarization

Fluorescence polarization was then employed to examine the drug-receptor interaction, its effects on the surrounding membrane fluidity, and the possibility of thermal interconversion of receptor subtypes. The degree of polarization for each probe was examined over the temperature

Figure 3.2. Temperature effect on the spectra of 12-AS ($\times 2\ 10^{-5}$ M) in F3/buffer. (A) Excitation spectra; Em. = 435 nm. (B) Emission spectra; Ex. = 364 nm. (---------- 44°C; ———— 10°C. Spectra of the blank at both temperatures is also shown. The blank contained 12-AS in buffer only.


FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
80
90
100
(A)
250
290
330
370
400
EXCITATION
(B)
440
480 nm
EMISSION

range of 44°C to 10°C, cooling instead of heating the samples slowly to measure subsequent values.

As can be seen in Figure 3.22, the degree of polarization, p, of DPH increased while the temperature was lowered. The degree of polarization, p, appears to be inversely related to the temperature of the system; in fact, statistical testing of the data using an analysis of variance indicates that the curve could be adequately fit by a straight line (Cook & Charnock, 1979). The 378 nm curve in this figure is given for comparison to that obtained with 358 nm excitation wavelength. The two curves are quite similar. Again p seems to be inversely related to the surrounding temperature. The slope of the curve corresponding to a wavelength of 378 nm is steeper.

The degree of polarization of 12-AS as a function of temperature is depicted in Figure 3.23. Again, p seems to be inversely related to the environmental temperature. The 383 nm curve is shown for comparison. Both curves can be described by a straight line without significant error. The degree of polarization of 12-AS was also observed using an excitation wavelength of 257 nm (Figure 3.24). The resulting values were negative in sign, and became more negative as the temperature was decreased. The increased variability of these data is probably due to the low intensity of the fluorescent light obtained when the more precise ratio mode was employed.

Having examined the polarization properties of DPH/membrane and 12-AS/membrane systems, possible drug effects were examined. The effect of histamine on DPH and 12-AS fluoresence polarization was observed.

Figure 3.22. Degree of polarization of DPH in F3 as a function of temperature. Em. = 429 nm. (o) Exc. = 358 nm; (●) Ex. = 378 nm. Results are expressed as Mean ± S.E.M. of 4 determinations.

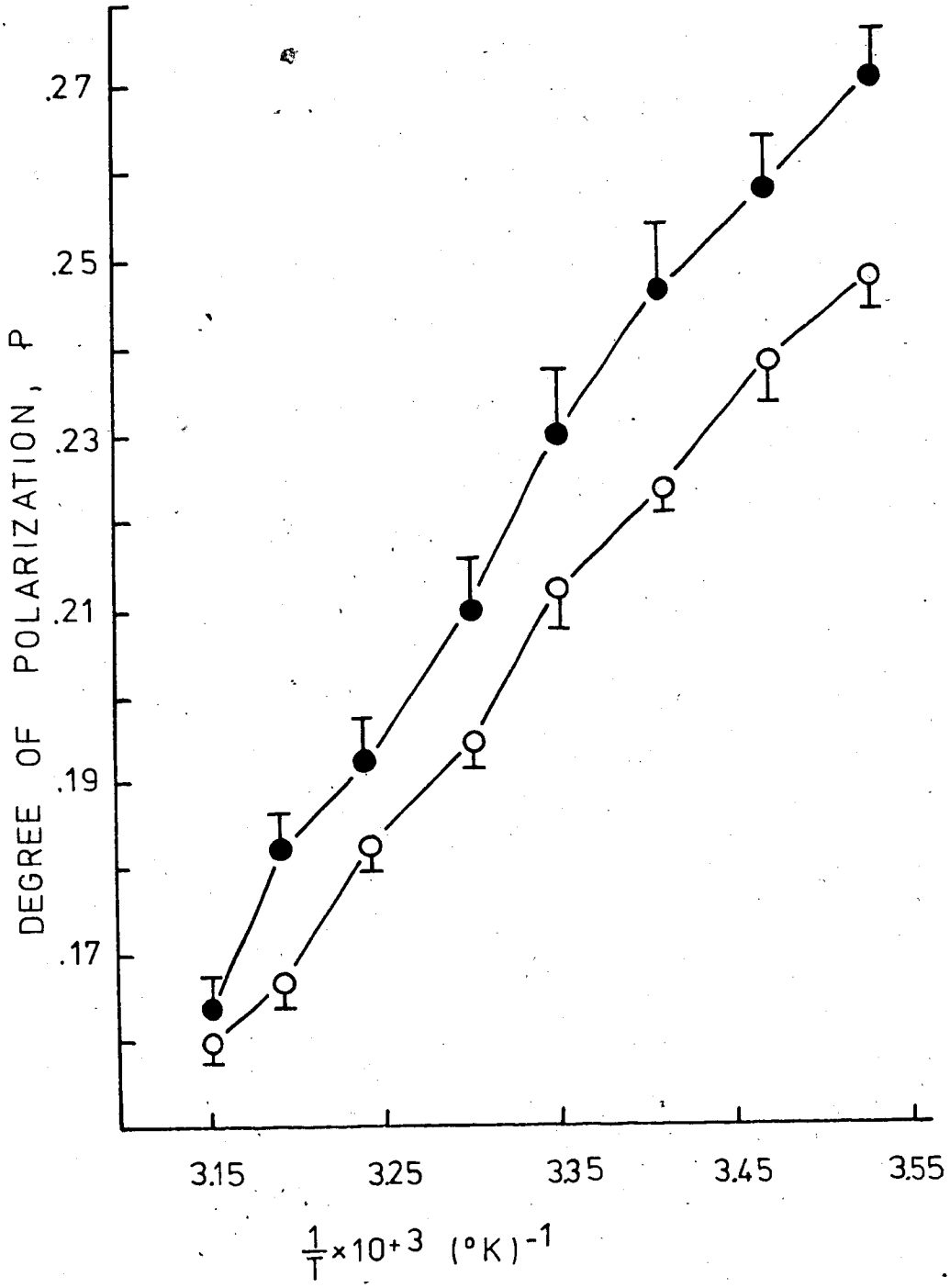

.27
.25
.23
.21
.19
.17
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.23. Degree of polarization of 12-AS in fraction F3 as a function of temperature. Em. = 437 nm. (○) Ex. = 364 nm; (●) Ex. = 383 nm. The points represent Mean ± S.E.M. of 4 determinations.

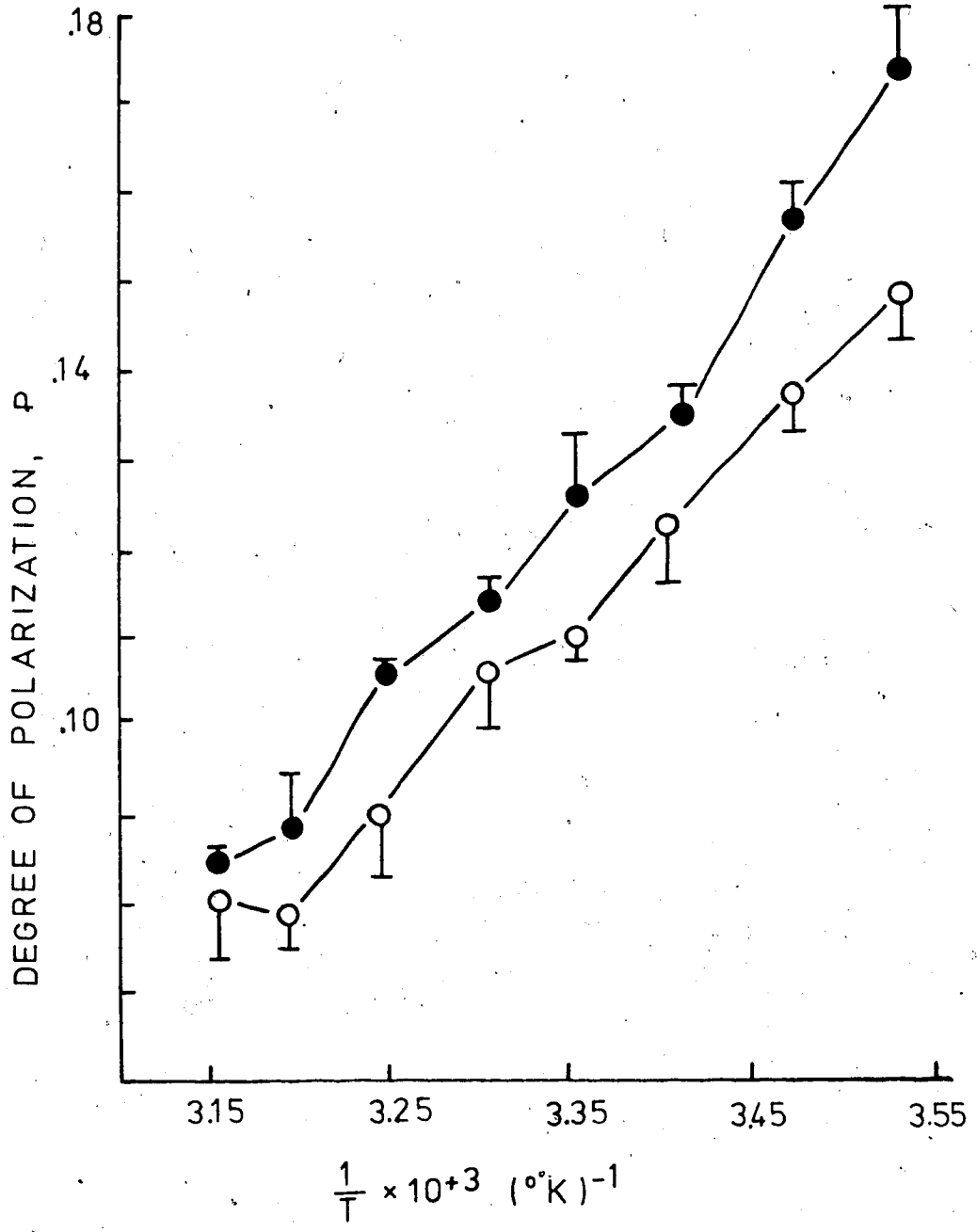
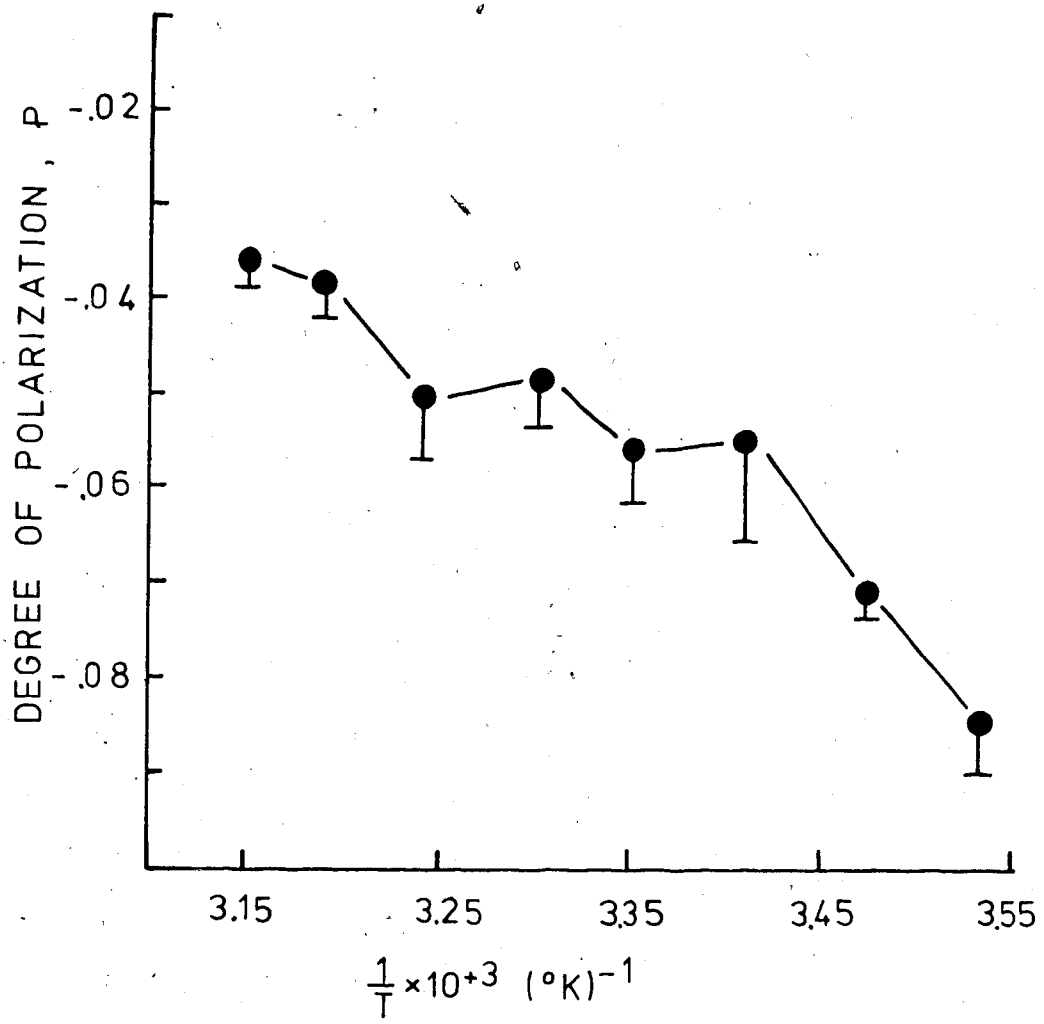

.18
.14
.10
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.24. Degree of polarization of 12-AS in F3 as a function of temperature. Ex. = 257 nm. Em. = 437 nm. Results are expressed as Mean ± S.E.M. of 4 determinations.


DEGREE OF POLARIZATION, P
-.02
-.04
-.06
-.08
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

The results with DPH are shown in Figure 3.25. The values of p were not significantly different from the membrane control. The slope of the curve was not altered in the presence of histamine. Upon examination of the 12-AS system, similar results were obtained (Figure 3.26).

Next, H_2 antagonists were added to the probe/membrane system to see if any effect such as alteration of membrane fluidity could be detected. Again, no change in either the values of p or the slope of the curve was observed for either of the two probes with either cimetidine or metiamide (Figures 3.27 and 3.28). The H_1 antagonists, chlorpheninamine and diphenhydramine, also had no apparent effect on either p or the slope for either the DPH curve or the 12-AS curve (Figures 3.29 and 3.30).

The adrenergic receptor system was also examined. First, adrenergic agonists were added. INA was used as a β-agonist while phenylephrine was employed as an α-agonist and NA as a mixed α and β physiological agonist. For both probes (Figures 3.31 and 3.32), p values at a given temperature and the slope of the curve remained unaltered. Use of the α-antagonist phentolamine and the β-antagonist propranolol left the values of P and the slope unaltered (Figures 3.33 and 3.34).

Thus, fluorescence polarization was unsuccessful as a tool for observing drug-receptor interaction and its effects at least using the F3 fraction. This also meant that the possibility of thermal interconversion of receptor subtypes in either the adrenergic or the histamine receptor system could not be examined.

Figure 3.25. Effect of histamine on the degree of polarization of DPH as a function of temperature. (X) membrane control; (▲) membrane plus 10^{-6} M histamine; (o) membrane plus 10^{-5} M histamine; (●) membrane plus 10^{-4} M histamine. Ex. = 358 nm; Em. = 428 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

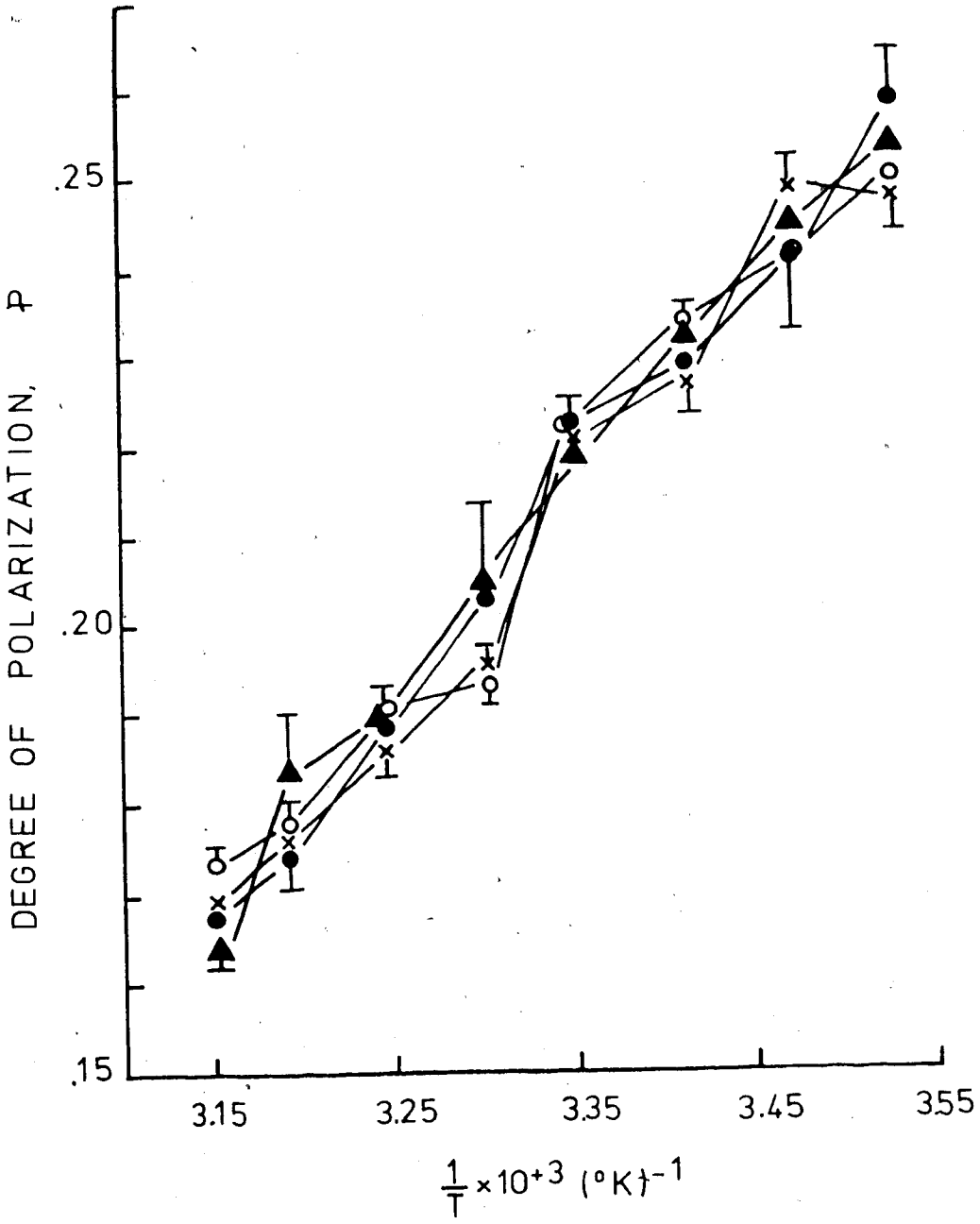

DEGREE OF POLARIZATION, P
.25
.20
.15
3.15
3.25
3.35
3.45
3.55
1/T x10+3 (°K)-1

Figure 3.26. Effect of histamine on the degree of polarization of 12-AS as a function of temperature. (X) F3 control; (O) F3 plus 10^{-6} M histamine; (▲) F3 plus 10^{-5} M histamine; (●) F3 plus 10^{-4} M histamine. Ex. = 364 nm; Em. = 437 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

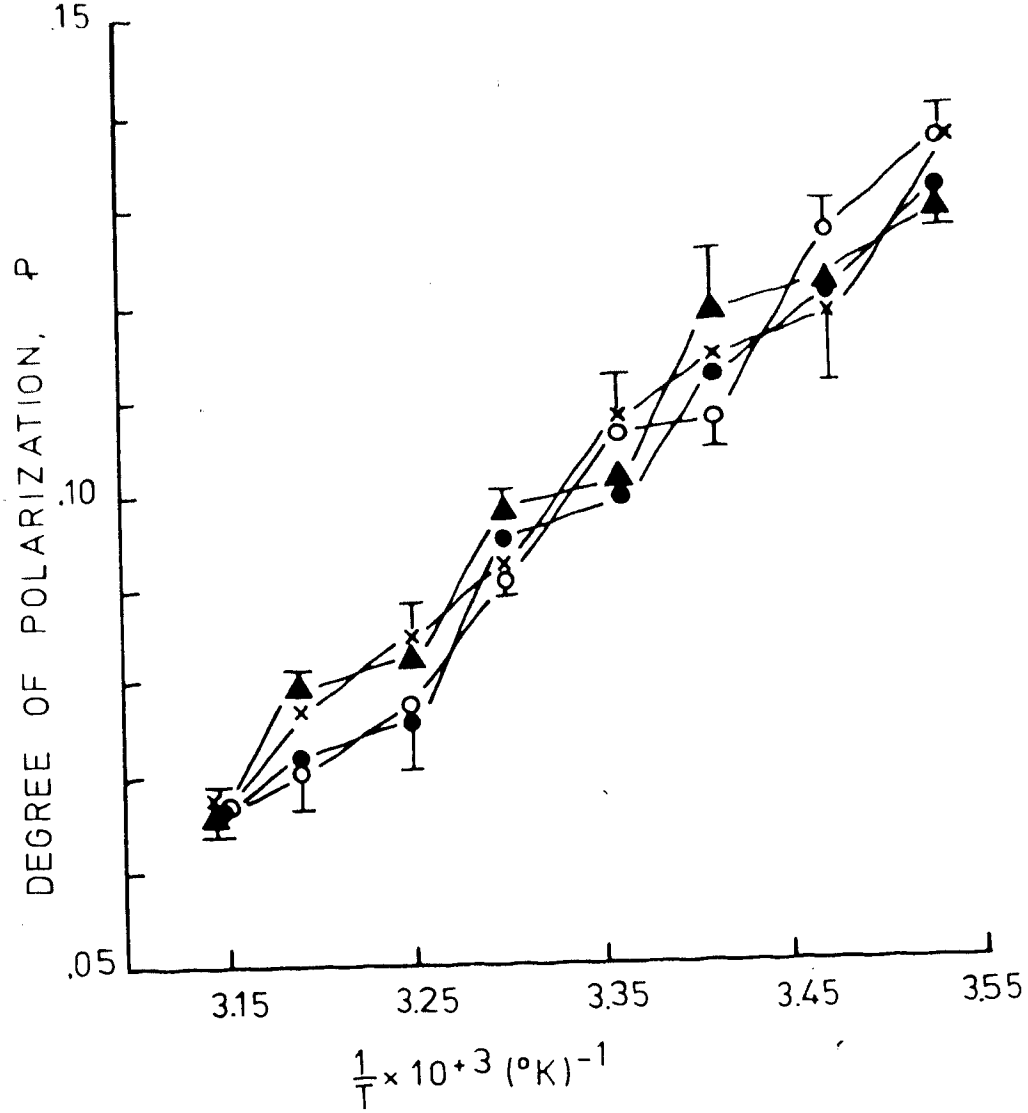

.15
.10
.05
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}\ (°K)^{-1}$

Figure 3.27. Effect of histamine H_2 antagonists (10^{-5} M) on the temperature dependent polarization curve for DPH. (X) membrane alone; (●) with cimetidine; (▲) with metiamide. Ex. = 358 nm; Em. = 428 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

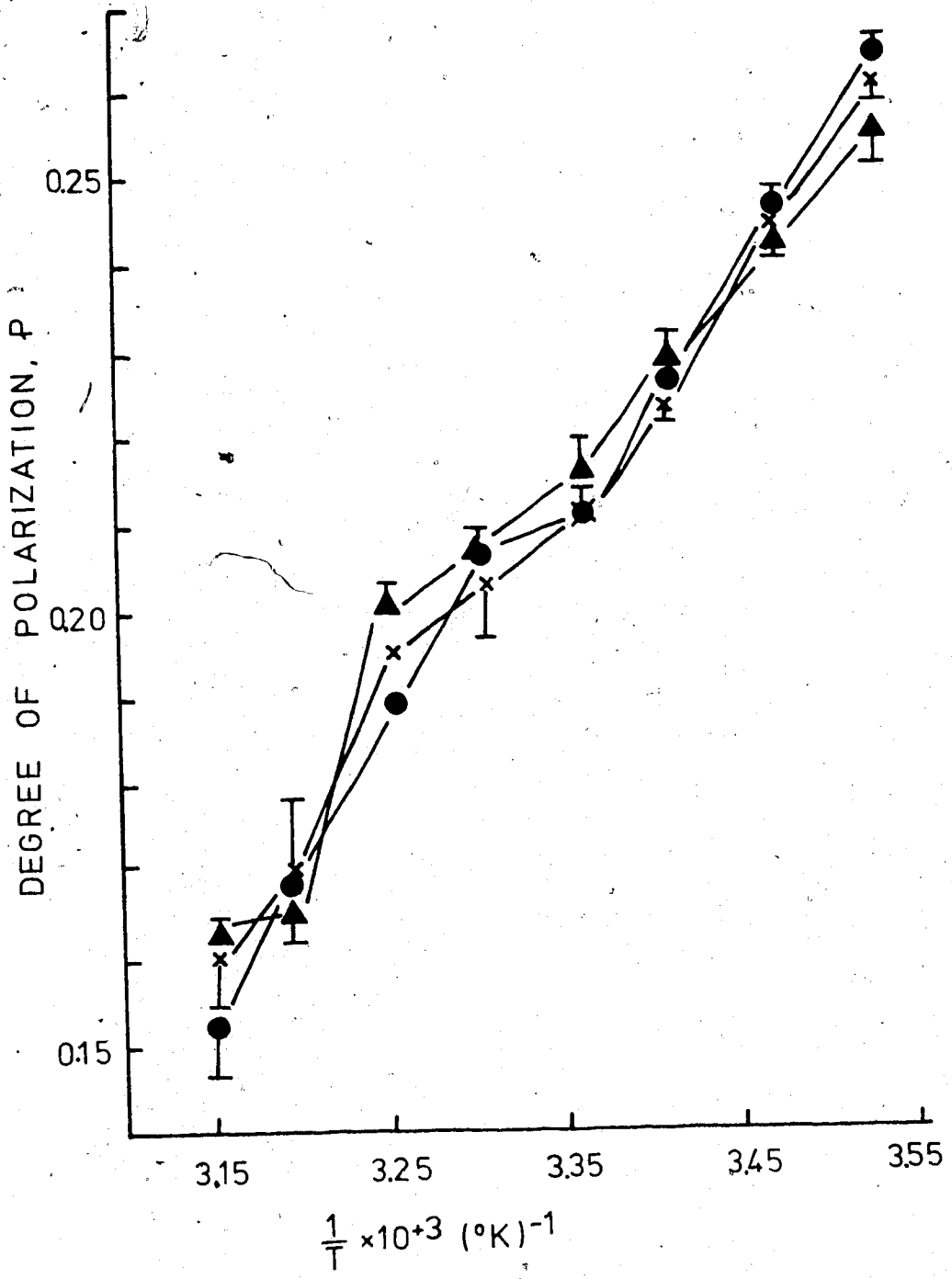
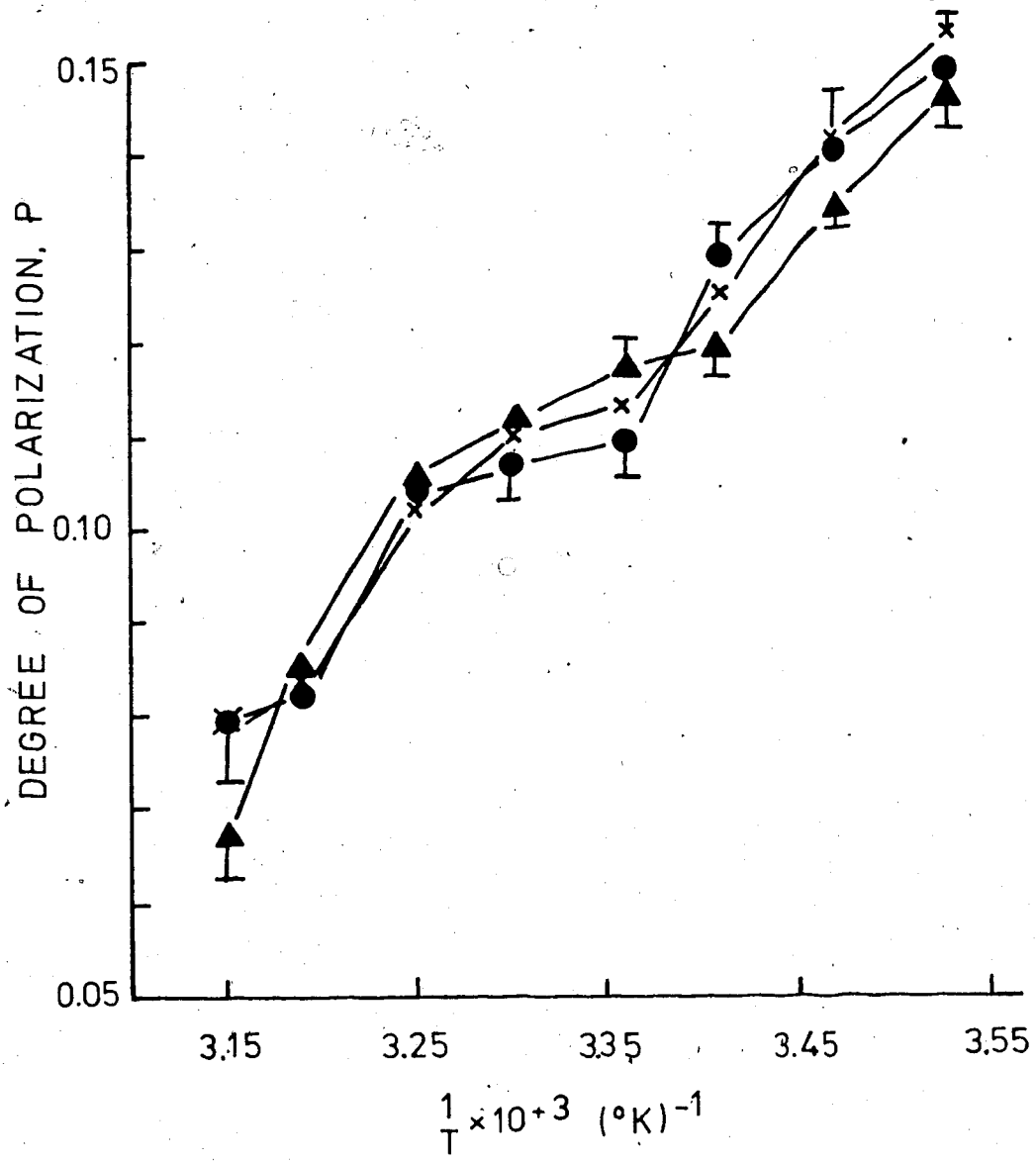

DEGREE OF POLARIZATION, P
0.25
0.20
0.15
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.28. Effect of histamine H_2 antagonists (10^{-5} M) on the temperature dependent polarization curve for 12-AS. (X) membrane alone; (●) with cimetidine; (▲) with metiamide. Ex. = 364 nm; Em. = 435 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.


0.15
0.10
0.05
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

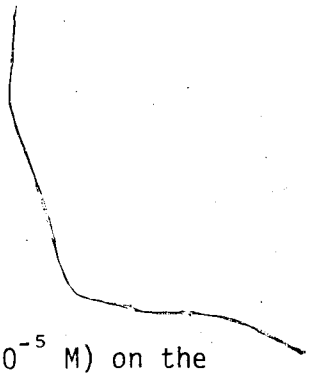


Figure 3.29. Effect of histamine H_1 antagonists.(10^{-5} M) on the temperature profile for DPH polarization curve. (X) membrane alone; (▲) with chlorphreniramine; (●) with diphenhydramine. Ex. = 358 nm; Em. = 429 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

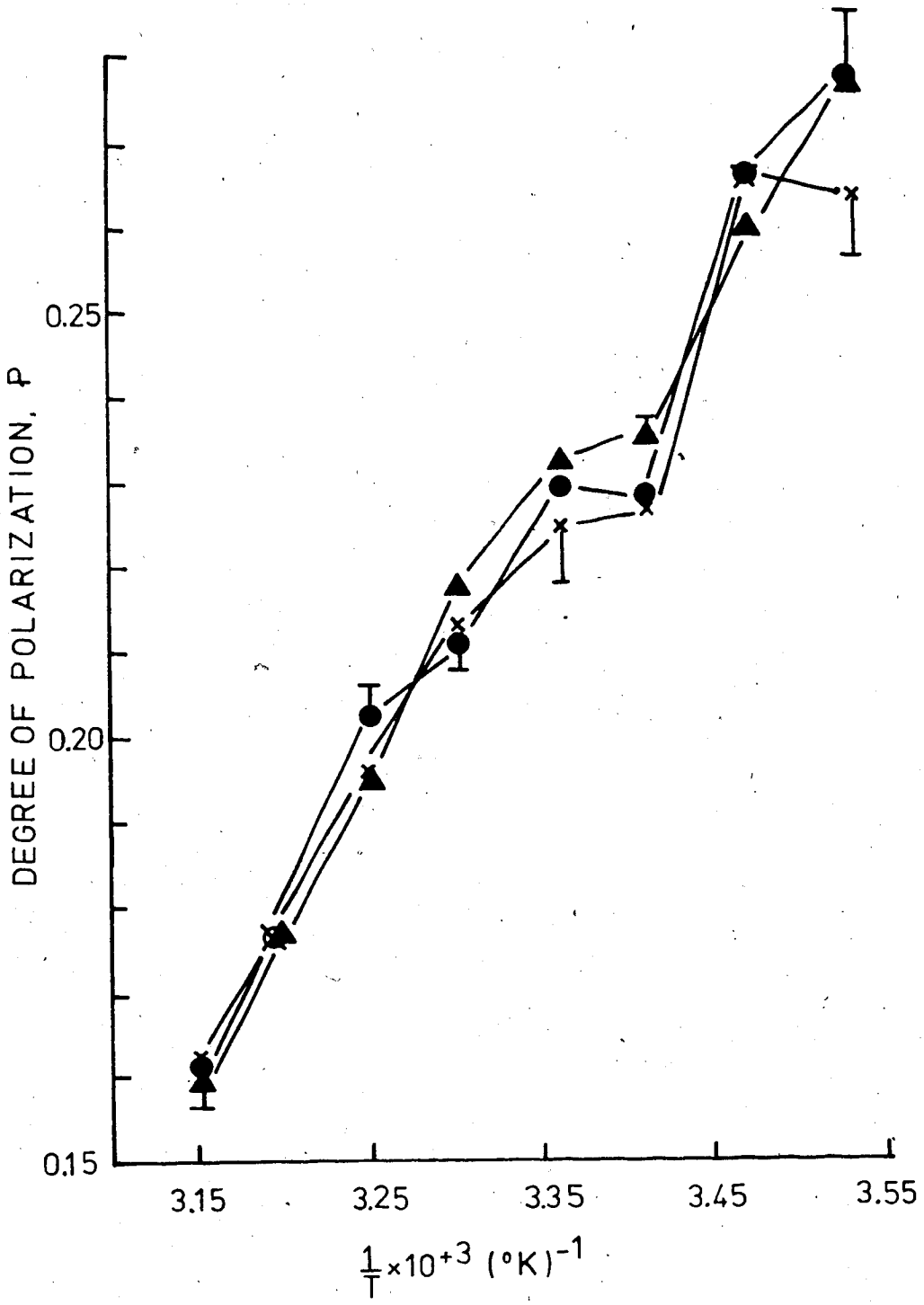

DEGREE OF POLARIZATION, P
0.25
0.20
0.15
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.30. Effect of histamine H_1 antagonists (10^{-5} M) on the temperature profile for 12-AS polarization curve. (○) membrane alone; (▲) with chlorphreniramine; (●) with diphenhydramine. Ex. = 363 nm; Em. = 437 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

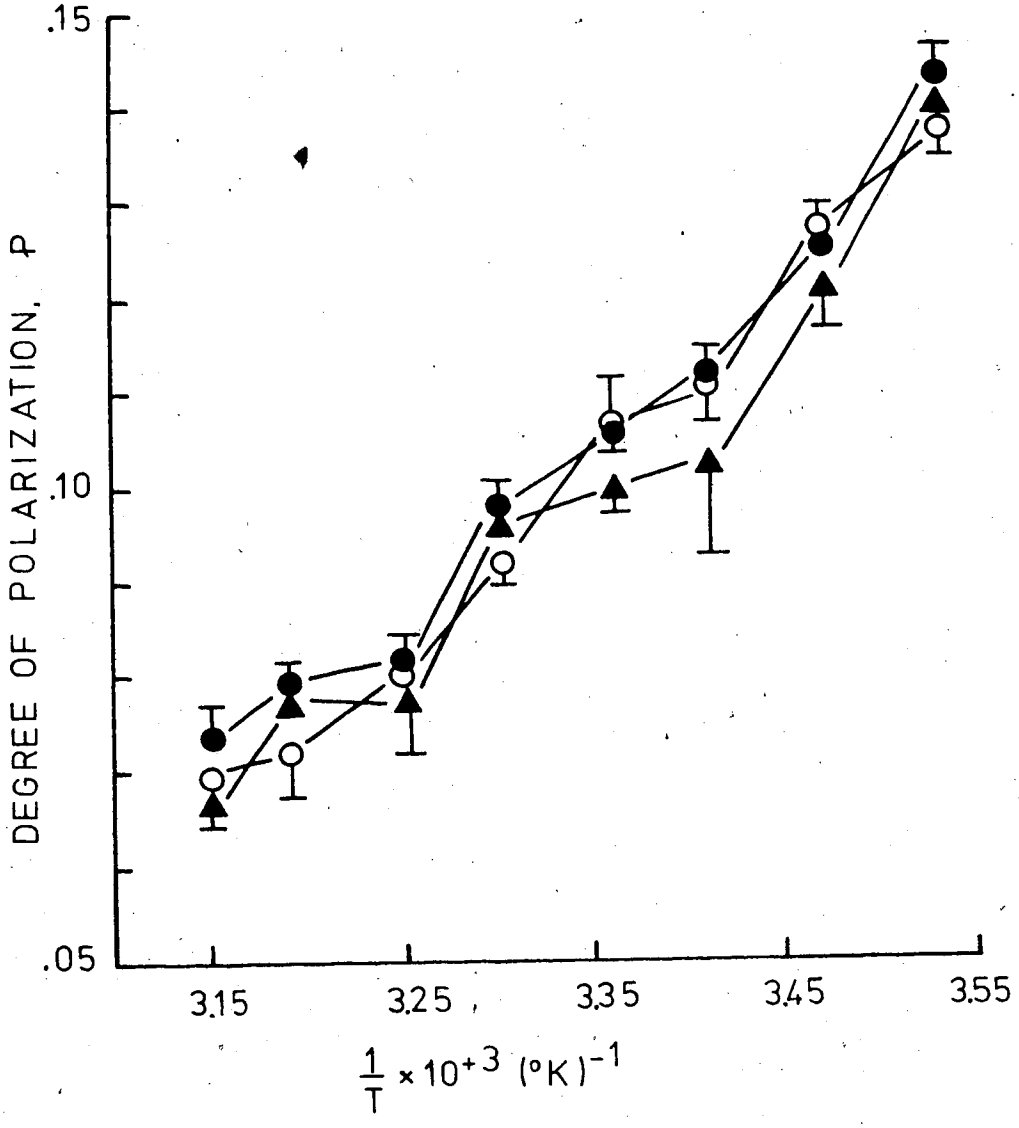

.15
.10
.05
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.31. Effect of adrenergic agonists on the degree of polarization of DPH as a function of temperature. Drug concentrations were all 10^{-5} M. (X) F3 alone; (●) F3 with INA; (O) F3 with NA: (▲) F3 with PE. Ex. = 359 nm; Em. = 428 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

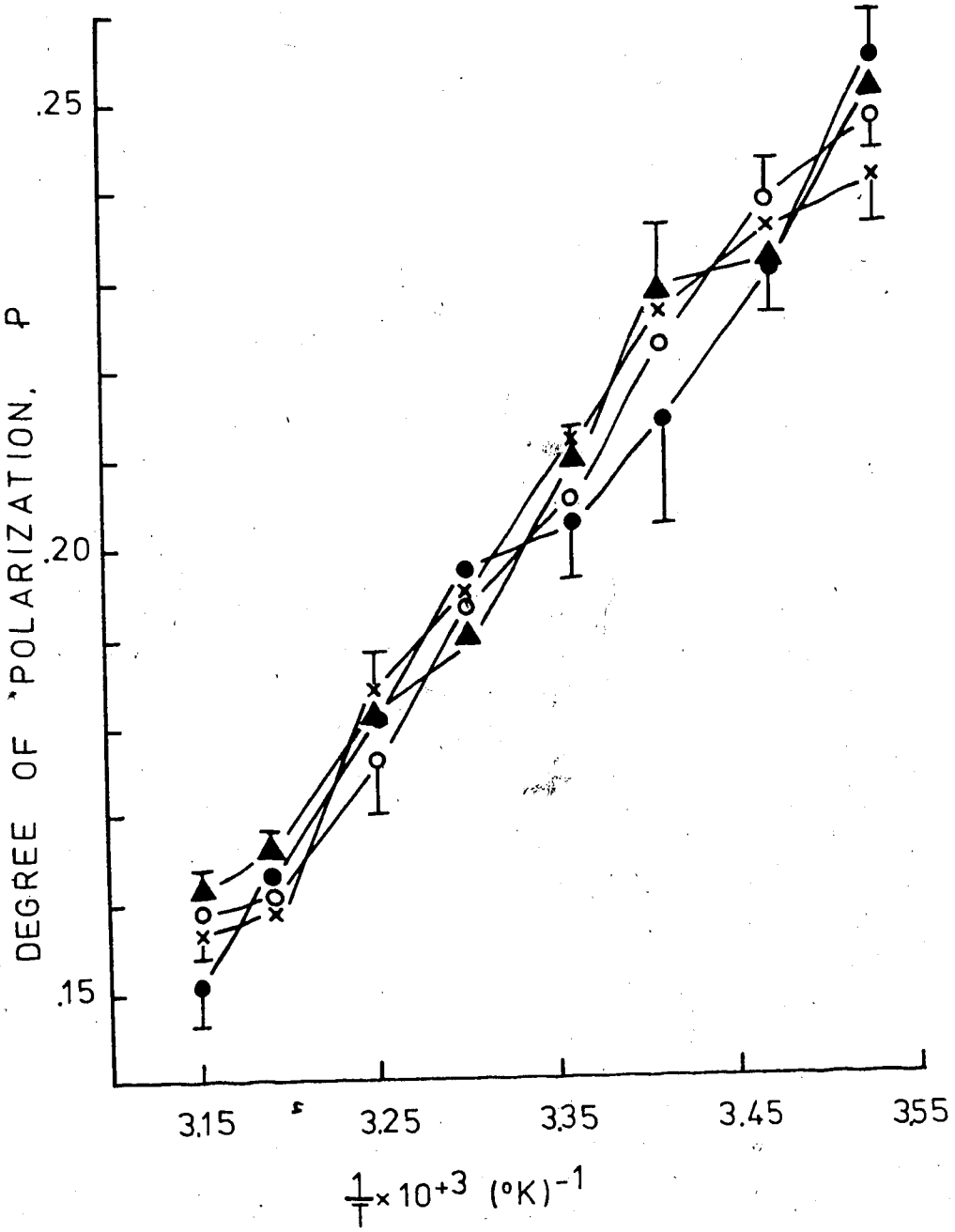

.25
.20
.15
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

Figure 3.32. Effect of adrenergic agonists on the degree of polarization of 12-AS as a function of temperature. Drug concentrations were all 10^{-5} M. (O) F3 alone; (X) F3 with INA; (▲) F3 with NA; (●) F3 with PE. Ex. = 364 nm; Em. = 437 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

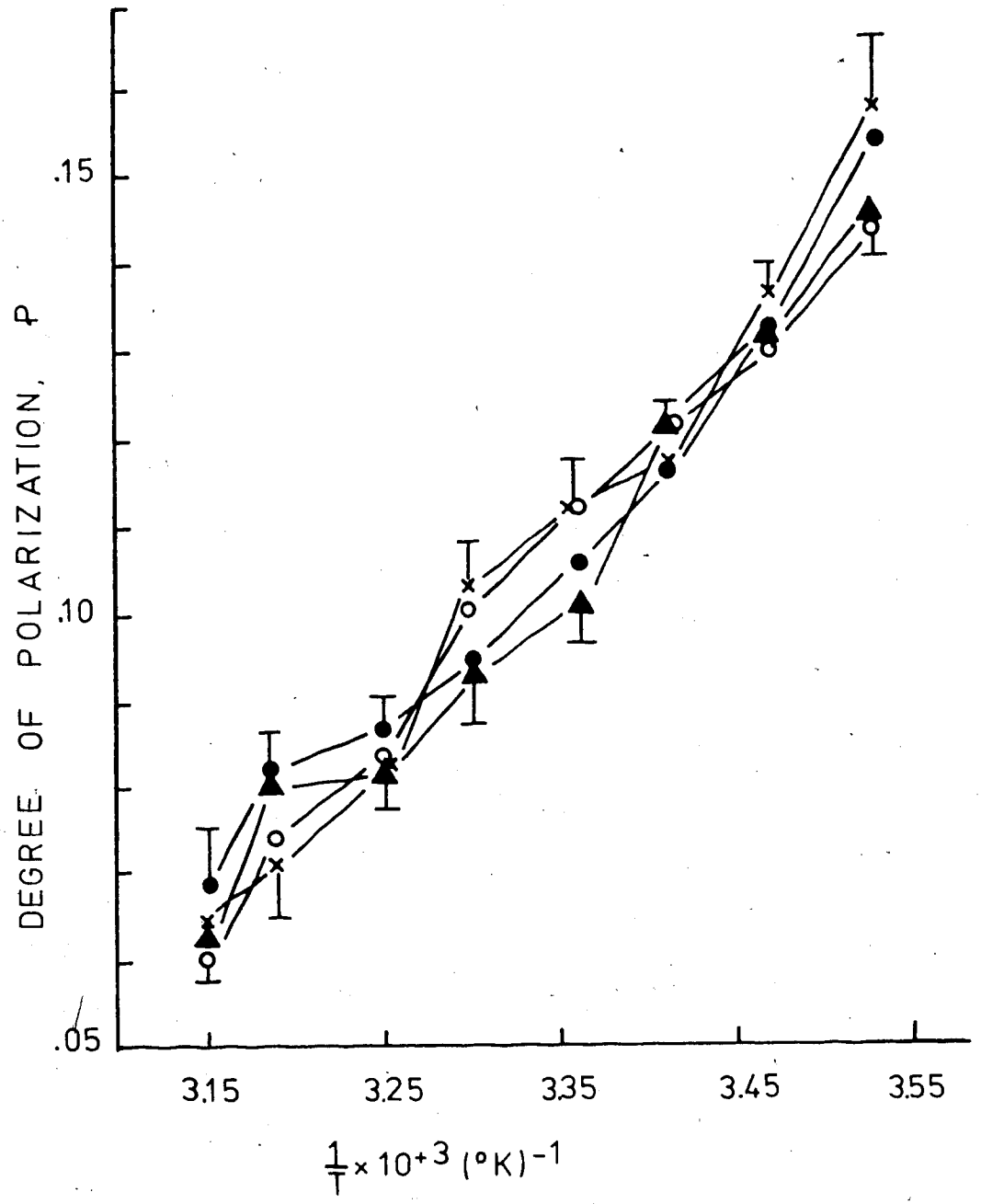

DEGREE OF POLARIZATION, P
.15
.10
.05
3.15
3.25
3.35
3.45
3.55
1/T x 10+3 (°K)-1

Figure 3.33. Effect of an α-adrenergic antagonist and a β-adrenergic antagonist on the degree of polarizaton of DPH as examined over the temperature range 44°C to 10°C. Drug concentrations were both 10^{-5} M. (O) F3 alone; (▲) F3 with phentolamine; (●) F3 with propranolol. Ex. = 358 nm; Em. = 427 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.

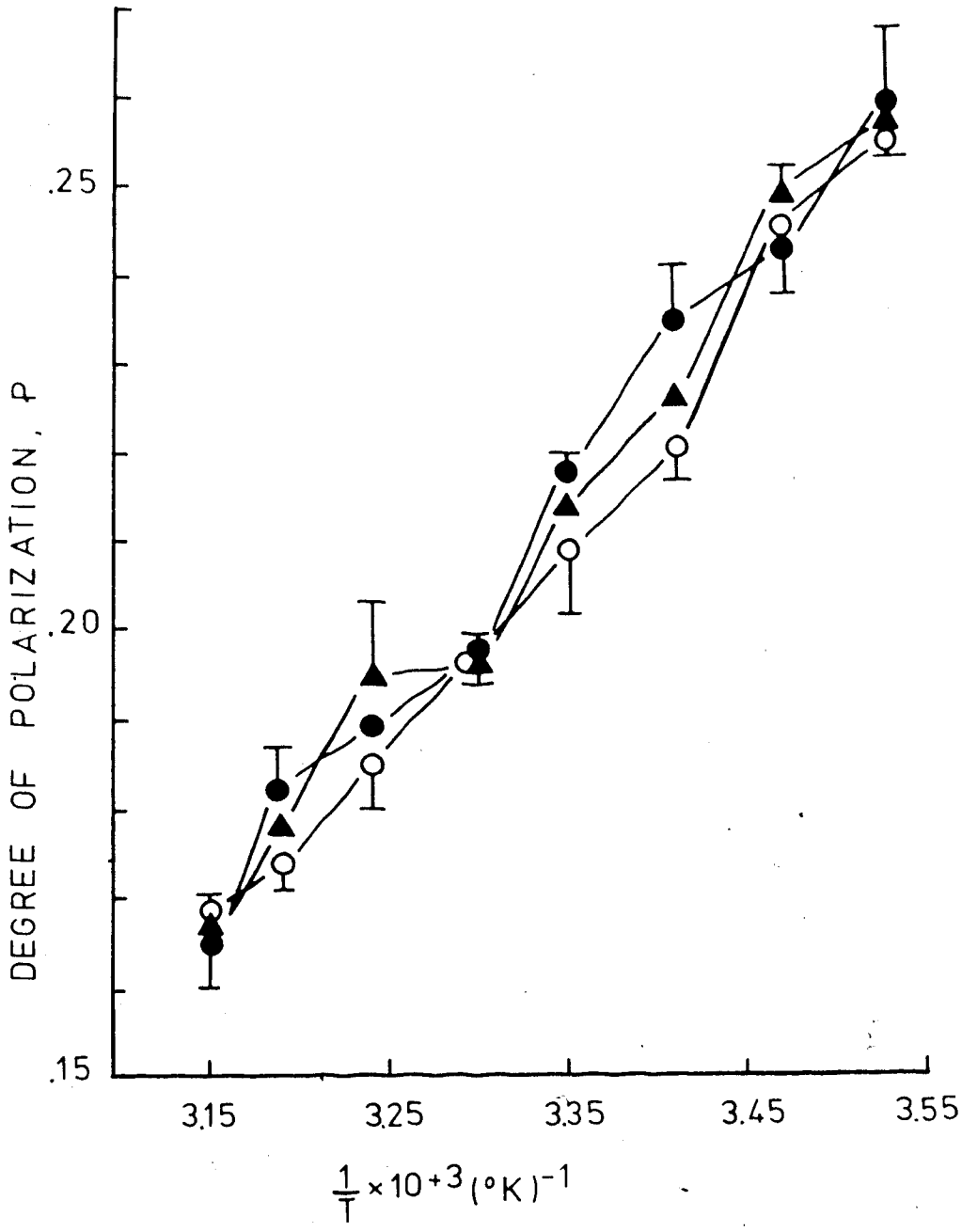
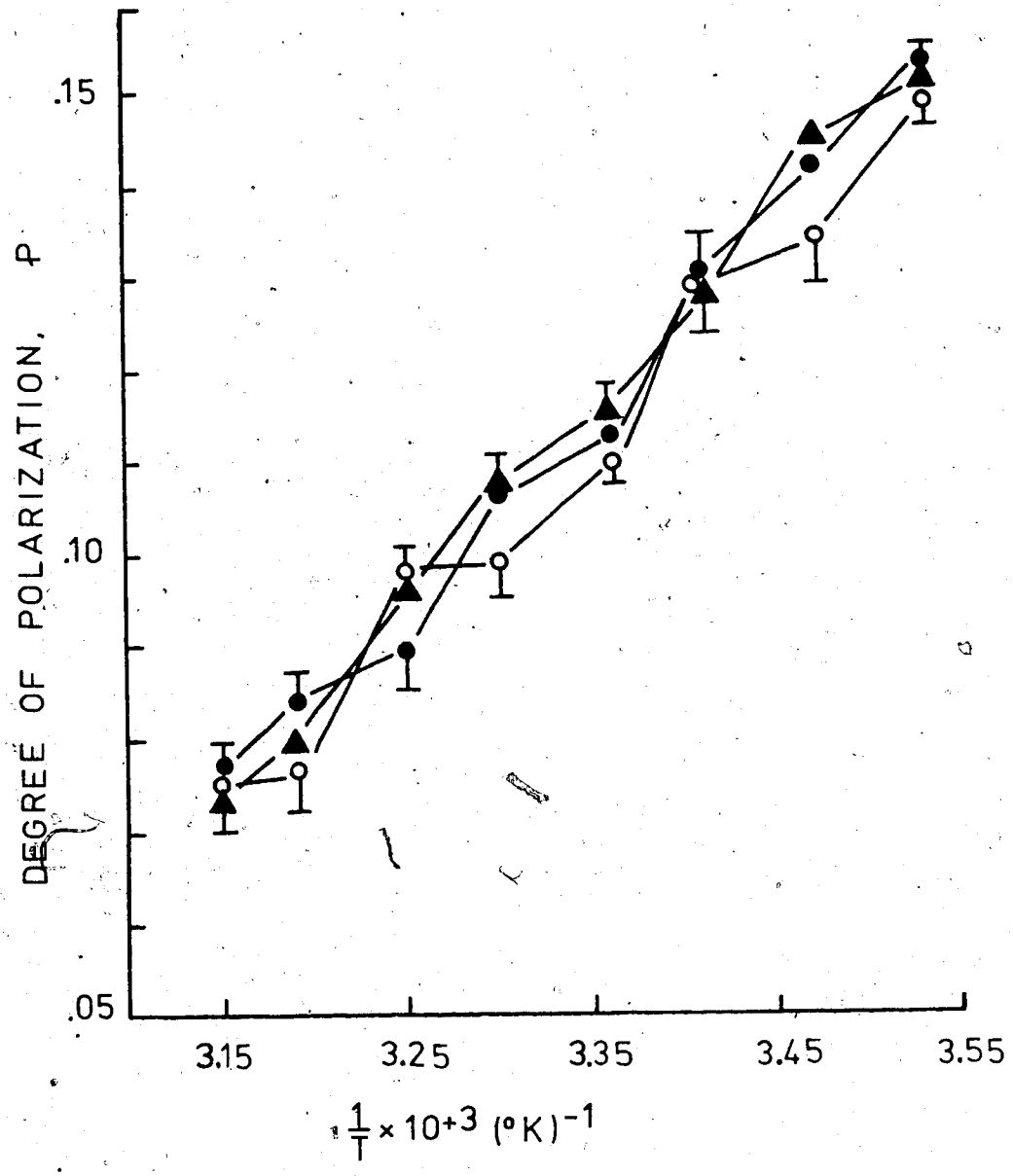

.25
.20
.15
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3} (^{\circ}K)^{-1}$

Figure 3.34. Effect of an α-adrenergic antagonist and a β-adrenergic antagonist on the degree of polarization of 12-AS as a function of temperature. Antagonist concentration were both 10^{-5} M. (●) F3 alone; (O) F3 with phentolamine; (▲) F3 with propranolol. Ex. = 364 nm; Em. = 437 nm. Results are expressed as Mean ± S.E.M. of 3 determinations.


.15
.10
.05
DEGREE OF POLARIZATION, P
3.15
3.25
3.35
3.45
3.55
$\frac{1}{T} \times 10^{+3}$ (°K)$^{-1}$

3.3 Tissue Studies

The effect of the two probes, DPH and 12-AS, as well as their corresponding solvents, THF and methanol, on INA and histamine responsiveness of right ventricular strips was ascertained. Neither DPH nor its solvent, THF appeared to have any significant effect on the histamine cummulative dose response curve (Figure 3.35). 12-AS and its solvent methanol gave similar negative results (Figure 3.36). Here, the sensitivity of the initial histamine dose response curves for the three groups was as variable as observed with treatment of these agents.

The effect of these agents on INA responsiveness was more difficult to determine. As well as initial variable responsiveness of individual tissues, the tissues appeared to sensitize to INA with time. This sensitization occured to varying degrees, thus giving rise to large standard errors. THF did not alter the INA cummulative dose response curve significantly (Figure 3.37). DPH, dissolved in THF, appeared to cause a depression of the curve although it is not significant due to the inconsistencies in dose response curves. Methanol did not seem to affect the INA dose response curve (Figure 3.38). 12-AS, dissolved in methanol, may increase the maximum but again the difference is not significant due to the large tissue variability.

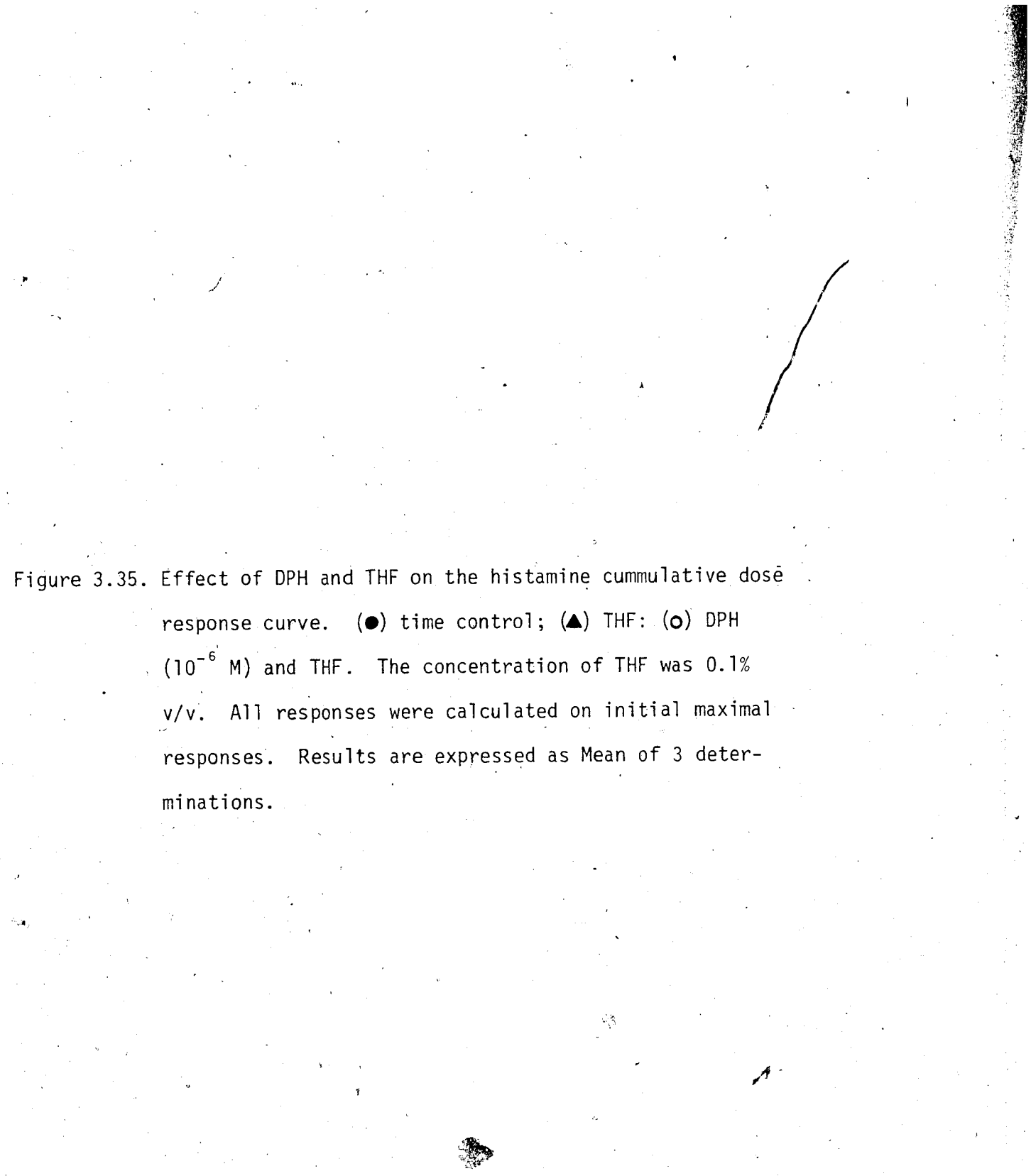


Figure 3.35. Effect of DPH and THF on the histamine cummulative dose response curve. (●) time control; (▲) THF: (o) DPH (10^{-6} M) and THF. The concentration of THF was 0.1% v/v. All responses were calculated on initial maximal responses. Results are expressed as Mean of 3 determinations.

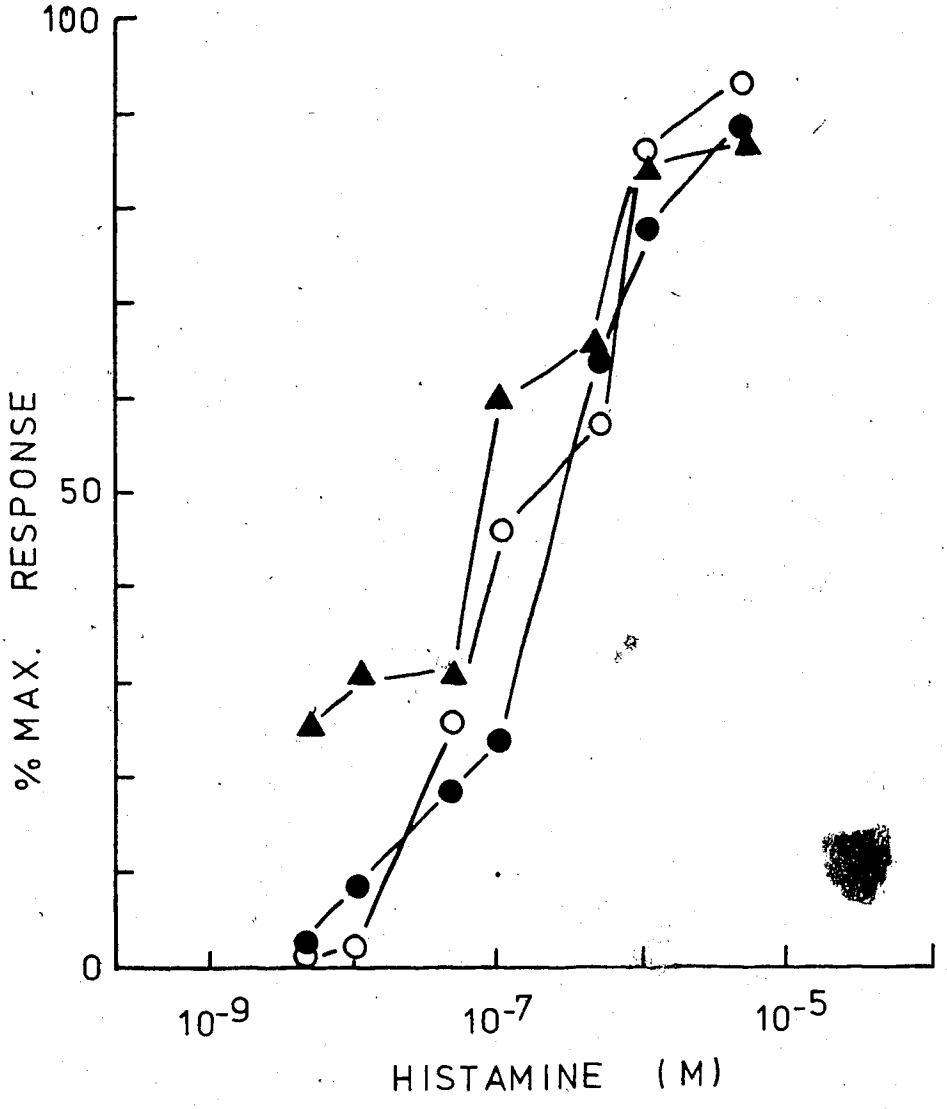

100
50
0
% MAX. RESPONSE
10^{-9}
10^{-7}
10^{-5}
HISTAMINE (M)

Figure 3.36. Effect of 12-AS and methanol on the histamine cummulative dose response curve. (●) time control; (▲) methanol; (o) 12-AS (2×10^{-6} M) and methanol. The concentration of methanol was 0.1% v/v. Results are expressed as Mean of 3 determinations.

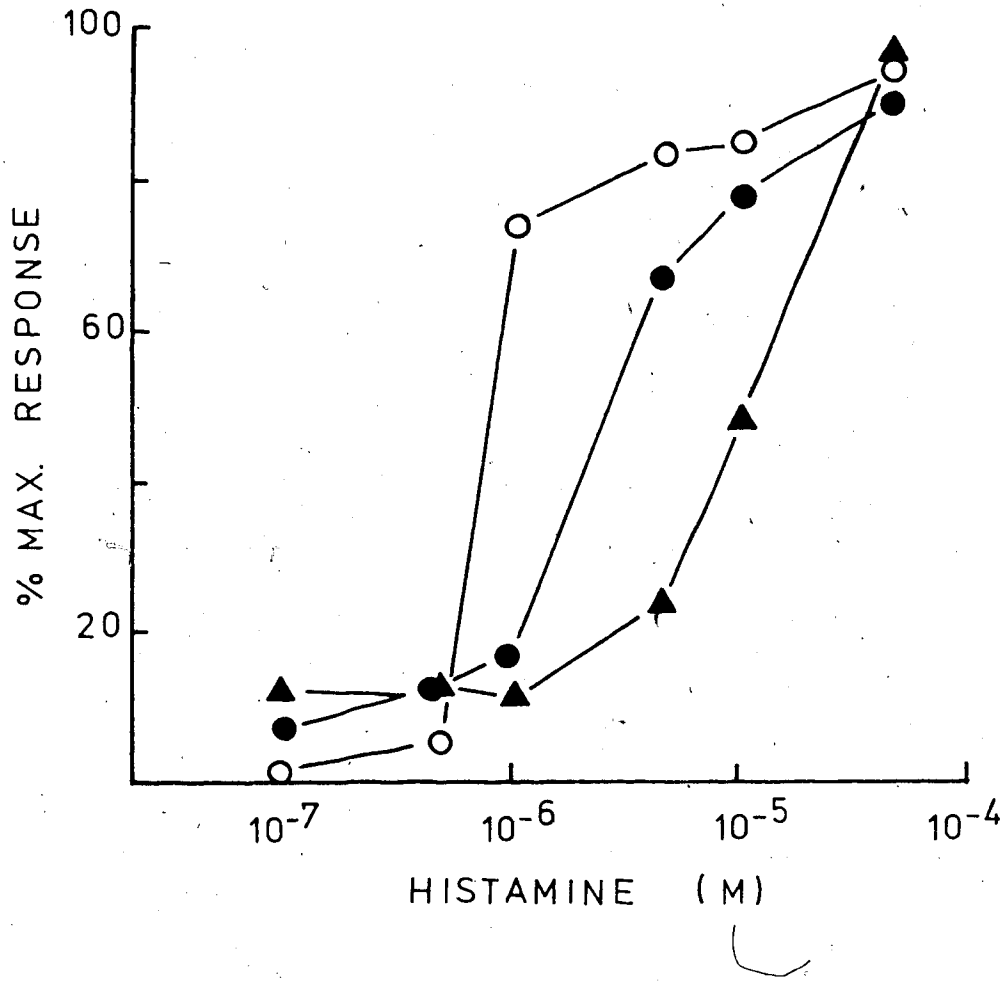

100
60
20
% MAX. RESPONSE
10^{-7}
10^{-6}
10^{-5}
10^{-4}
HISTAMINE (M)

Figure 3.37. Effect of DPH and THF on the INA cummulative dose response curve. (o) time control; (●) THF: (▲) DPH (10^{-6} M) and THF. The concentration of THF was 0.1% v/v. Results are expressed as Mean of 3 determinations.

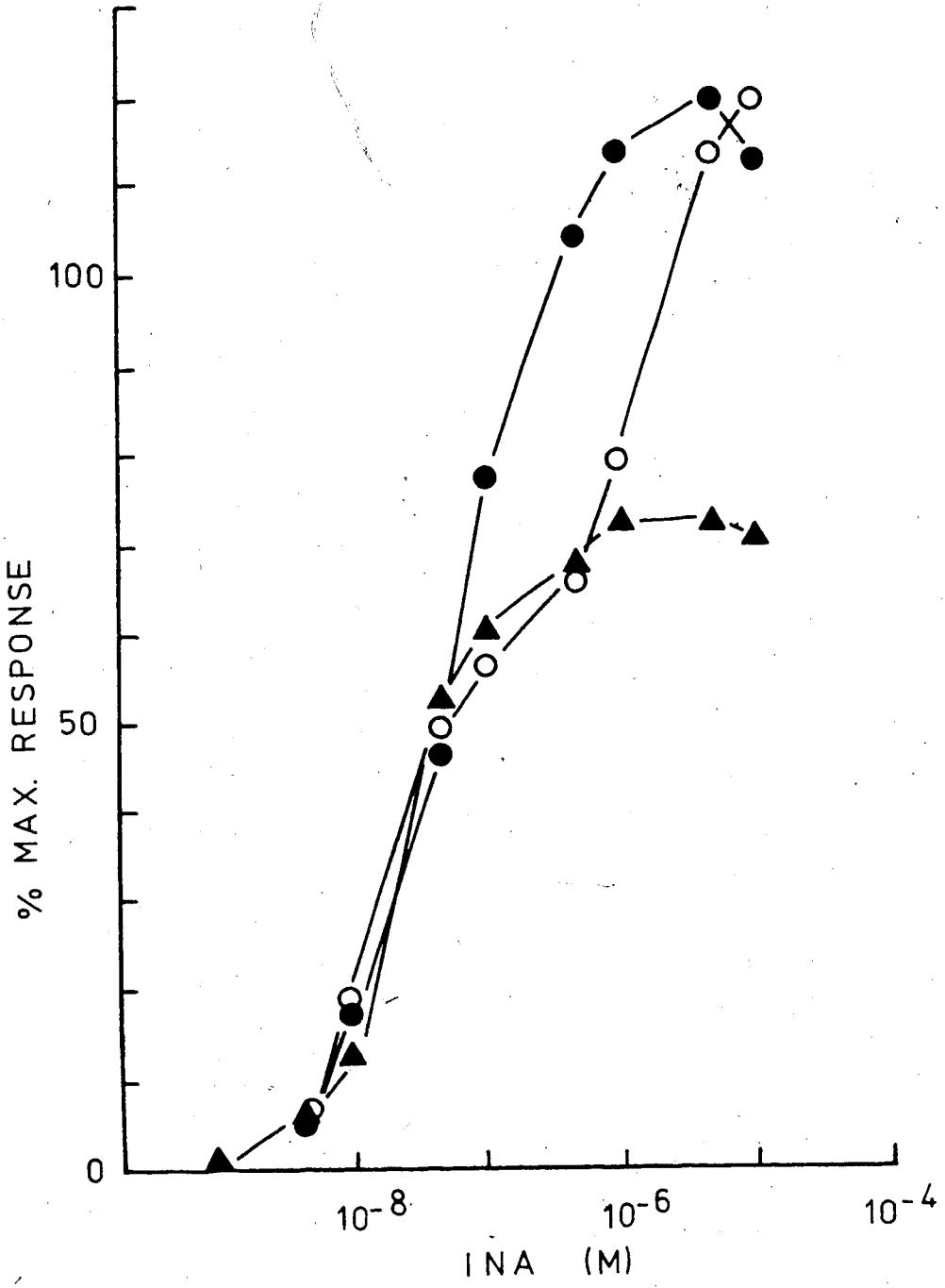
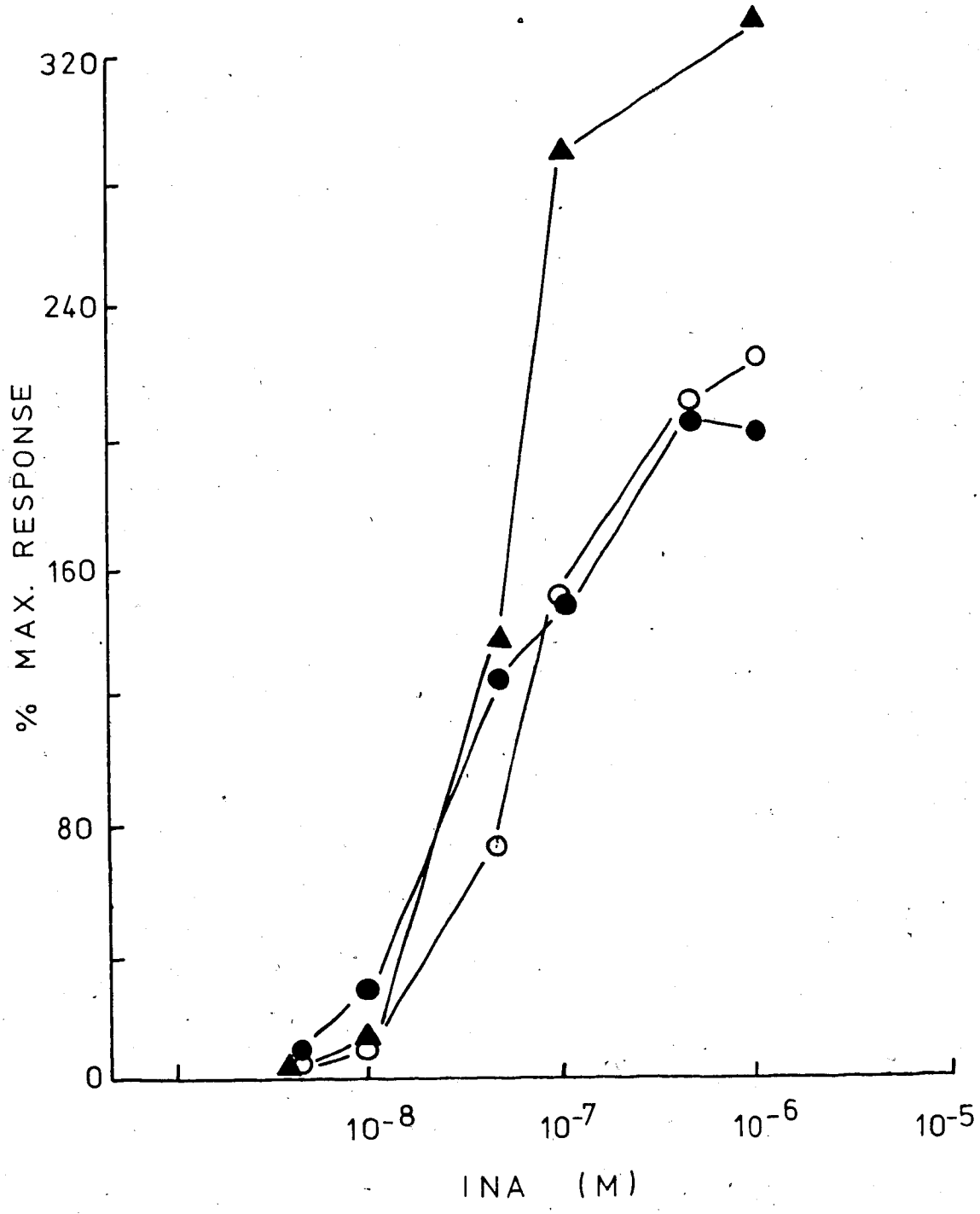

100
50
0
% MAX. RESPONSE
10^{-8}
10^{-6}
10^{-4}
INA (M)

Figure 3.38. Effect of 12-AS and methanol on the INA cummulative dose response curve (●) time control; (○) methanol, (▲) 12-AS (2×10^{-6} M) and methanol. The concentration of methanol was 0.1% v/v. Results are expressed as Mean of 3 determinations.


320
240
160
80
0
% MAX. RESPONSE
10-8
10-7
10-6
10-5
INA (M)

Chapter 4

DISCUSSION

4.1 Purification and Characterization of Cardiac Sarcolemma Enriched with Histamine H_2 and β-Adrenergic

Partial purification of the cardiac sarcolemma from the guinea pig was achieved in fraction F3. This fraction was found to be purified over 10 fold with respect to adenylate cyclase. It contained both histamine H_2 receptors and β-adrenergic receptors, as measured by histamine and INA stimulation of adenylate cyclase. Evidence by a number of groups strongly suggest that histamine H_2 and β-adrenergic actions on the heart are mediated by stimulation of adenylate cyclase and a subsequent rise in intracellular cAMP levels (Klein and Levey, 1971; Kukovetz et al., 1973; Martinez and McNeill, 1975; McNeill and Muschek, 1973; Reinhardt et al., 1977; Tada et al., 1975; and Verma and McNeill, 1976). It was for this reason that stimulation of adenylate cyclase via agonists was employed to detect the presence of both histamine H_2 and β-adrenergic receptors.

Fraction F3 was enriched, when compared to the homogenate, about four fold in $(Na^+ + K^+)$-ATPase and about three fold in alkaline phosphatase, the two putative plasma membrane markers. There appeared to be reduced contamination of the inner mitochondrial membrane in fraction F3 as measured by the enzyme distribution of succinate dehydrogenase while there was contamination by the outer mitochondrial membrane in this fraction as estimated by the two enzymes, rotenone-insensitive NADH cytochrome c reductase and monoamine oxidase. Characterization by electron microscopy showed F3 to be vesicular in nature, with a range of 78 to 200 nm in the diameter.

A comment on the validity of enzymes as specific markers for particular cellular organelles and membranes is appropriate here. Good reviews of the problem have been published by de Duve (1971), DePierre and Karnovsky (1973) and Solyom and Trams (1972). Initially the localization of an enzyme to a membrane was obtained using histochemical techniques. This was usually done in liver tissue for a number of enzymes. Most other enzymes were said to be membrane markers when they were found to co-migrate, or co-distribute, with enzymes shown to be located on that particular membrane by histochemistrical techniques. This is supportive evidence but is not conclusive evidence. Furthermore, results from one or two tissues in one species have been assumed to be representative, and thus, the conclusions have been accepted as being universal that is, applying to many different tissues as well as species. Often circular arguments are employed such as "this is the plasma membrane fraction and thus this enzyme which is found in the fraction is a marker for the plasma membrane" with little demonstration by other techniques that the fraction is indeed purified plasma membrane.

In this particular case, $(Na^+ + K^+)$-ATPase in the guinea pig heart has recently been demonstrated on the plasma membrane and the t-tubular membrane using a histochemical technique (Asano et al., 1980) and thus is probably a valid marker for the sarcolemma in the guinea pig heart. However, the use of alkaline phosphatase as a sarcolemma marker in this tissue appears to be questionable. Although it has been employed in the past (Saccomani et al., 1974; and Wang et al., 1977). In all sucrose gradient fractions and even the supernatant, this enzyme was found to be

purified to the same extent. Fractions F1 and F2 and the supernatant contain mostly nonmembrane protein. Thus, either alkaline phosphatase is not solely located on the plasma membrane or it is only loosely associated with the plasma membrane and may easily dissociate and reassociate with other membranes during the isolation proceedure.

No enzyme has been found to be adequate as a marker for sarcoplasmic reticulum (SR). Glucose-6'-phosphatase, commonly used as a marker for endoplasmic reticulum in liver tissue and some other tissues, is either absent or present in barely detectable amounts in heart tissue. However, a few groups have employed this enzyme as a cardiac SR marker (Bers, 1979; St. Louis and Sutakre, 1976a). More recently, NADPH cytochrome c reductase has been employed as such a marker (Bers, 1979; Paris et al., 1977; Wang et al., 1977). This results from its presence in heart tissue and its use as an endoplasmic reticulum marker in liver cells and in smooth muscle. There, however, some report of its presence on Golgi membranes (Evans, 1978). Ca^{2+}-ATPase is also often employed as a cardiac sarcoplasmic reticulum marker (Alstyne et al., 1979; Besch et al., 1977; Jones et al., 1979, Saccomani et al., 1974) although there is some suggestion that it is also present on the sarcolemma (Bers, 1979; Dhalla et al., 1976; Hui et al., 1976; St. Louis and Sulakhe, 1976b).

In this study, NADPH cytochrome c reductase was used to estimate the extent of sarcoplasmic reticulum contamination. The F3 fraction was found to show some contamination by this enzyme. Its presence in the sarcolemma fraction has been observed and its distribution on the

sucrose gradient (Figure 3.10) appears similar to that reported elsewhere (Bers, 1979; Matlib et al., 1979; Paris et al., 1977; Wang et al., 1977).

Mitochondrial contamination has most often been determined using succinate dehydrogenase or cytochrome c oxidase. These two enzymes are present on the inner membrane of the mitochondria and are thus only valid markers for mitochondria so long as the organelles stay intact (Comte and Gautheron, 1978a, b; Evans, 1978; Matlib et al., 1979; Sottocasa et al., 1967 a, b). The outer membrane of the mitochondria may be stripped off during the isolation proceedure (Evans, 1978; Neville, 1975) and therefore this membrane should be regarded as a separate entity when determining contamination by other subcellular organelles and membranes. Only recently have the two enzymes, monoamine oxidase (MAO) and rotenone-insensitive NADH cytochrome c reductase, been used to quantify the outer mitochondrial membrane. Often these two enzymes were found to contaminate the sarcolemma fractions to varying degrees (Alstyne et al., 1979; Bers, 1979; Kwan et al., 1979; Matlib et al., 1979; Wang et al., 1979). Matlib et al. (1979) have suggested that neither monoamine oxidase nor rotenone-insensitive NADH cytochrome c reductase may be considered to be solely localized to the outer membrane of the mitochondria but may also be present on other subcellular membranes.

Thus the choice of which enzymes to use as specific membrane markers for a particular tissue and species is important. Markers for cardiac sarcolemma are $(Na^+ + K^+)$-ATPase and adenylate cyclase although

it may be more appropriate to employ the hormone stimulated adenylate cyclase instead of the basal activity. Inner mitochondrial membrane contamination seems to be adequately determined by succinate dehydrogenase or cytochrome c oxidase. No enzyme appears to satisfactorily serve as an indicator for cardiac sarcoplasmic reticulum. Similarly an enzyme has not been found to be solely located on the outer membrane of mitochondria. Thus with these two membrane systems, the presence of enzymes can only serve as a rough indication that the membranes may be present. Enzymes must be found to be localized to each of the sarcoplasmic reticulum and the outer mitochondrial membrane of cardiac tissue by histochemical nears before further purification and characterization can proceed.

With the reservations discussed above it seems clear that the fractions labelled F3 is most appropriate for further experiments requiring isolated sarcolemma. One additional point which should be discussed, however, is the rationale for the subdivision of what looks like a rather broad band into the two fractions F3 and F3 (Figure 3.4). The reasons for this somewhat arbritrary division are discussed below.

First the two bands had slightly different appearances on the sucrose gradient. F3 was more diffuse while F4 was more opaque and concentrated in nature. Also, their microscopic appearances were different (Figure 3.13 and 3.14). F3 contained vesicles of 78 to 200 nm in diameter, while in F4, the vesicles were of a much larger range - 65 to 375 nm. There was also some membrane "sheets" (unsealed membranes), an occasional intact mitochondrion and some denser vesicles (darker in

terms of the electron micrographs) in fraction F4. There was an occasional appearance of nonmembrane protein as seen in fractions F1 and F2 (Figure 3.11 and 3.12).

More importantly, although both F3 and F4 were found to contain histamine receptors, only F3 was found to contain β-adrenergic receptors capable of stimulating adenylate cyclase. Similarly the enzymatic profiles were somewhat different. Succinate dehydrogenase and $(Na^+ + K^+)$-ATPase were found in higher levels in F4. There was also slightly more NADPH cytochrome c reductase in F4. Thus on the basis of both microscopic appearance and biochemical characterization, the two fractions F3 and F4 are different and their division is well justified.

4.2 Fluorescence Studies

Two probes, DPH and 12-AS, were employed in the fluorescence studies. Both tend to favour the deep hydrophobic region of the bilayer. Equilibration of the probes with the F3 fraction was achieved within 25 minutes for 12-AS and 40 minutes for DPH at 44°C, as measured by stability of the fluorescence intensity. These values were then used as minimal incubation times for subsequent experiments.

4.2.1 Fluorescence Spectra

The characteristic fluorescent spectra of both probes in the F3 fraction was examined. For the lipophilic probe, DPH, there were two excitation maxima, 358 and 378 nm, and for each excitation wavelength, the emission maximum remained the same at 428 nm. The two excitation maxima probably reflect differences in the energy levels of two vibrational levels of the excited probe molecules. The emission maximum will remain constant irrespective of the wavelength of the exciting light. It corresponds to the release of light energy due to the transition of the excited probe molecule from the lowest vibrational level of the first singlet excited state to the ground state.

In the case of 12-AS, the two excitation maxima of 364 nm and 383 nm and the same emission maximum of about 437 nm, observed in the presence of the F3 fraction, can be explained as above. The other observed excitation maximum at 257 nm is well separated from the rest of the excitation spectrum. This isolated peak is probably due to a transition to a much higher energy level (the anthracene moiety can strongly absorb light at approximately 255 nm although the wavelength is strongly

substituent and solvent dependent). It may also be the result of energy transfer from either tryptophan or tyrosine residues of membrane proteins to nearby 12-AS molecules, but the excitation maximum is usually found between 275 and 295 nm.

The temperature dependence of the excitation and emission spectra for 12-AS and DPH was examined by comparing the spectra at 44°C and 10°C. For 12-AS, the fluorescence intensity was greatly increased for all three excitation maxima. This can be explained as follows: a reduction in the temperature decreases the frequency of collisions between the excited fluorophore and the surrounding solvent molecules (as in the case of increased viscosity) and therefore lowers the probability of external nonradiative conversion with the result that the quantum yield is increased.

For the probe, DPH, only a slight increase in fluorescence intensity was observed upon lowering the temperature to 10°C. It is possible that collision dependent processes are not of great importance as competing factors. On the other hand, the two excitation maxima, 358 nm and 378 nm, were shifted about 2 to 3 nm to the right (a red shift) while there was no apparent shift in the 12-AS spectra. The spectral shift observed for DPH can be interpreted as a slight alteration in the energy difference between ground and excited states.

Next, possible effects of various drugs on the DPH and 12-AS spectra was studied. A number of other researchers have observed specific drug-induced spectral changes of biomembranes (Elferink, 1977; Radda, 1975b; Rooney et al., 1979; Spero, 1978). First, the question of shifts in spectral maxima was answered for both the probes. Neither

histamine nor the β-adrenergic agonists INA and NA (which also has α-agonist activity) had any effect. Next the H_2 antagonists, metiamide and cimetidine, and the β-antagonist, propranolol, were tested and again found to have no apparent action on the excitation and emission maxima of either DPH or 12-AS. The histamine H_1-antagonists, chlorpheniramine and diphenhydramine, the α-agonist PE and the α-antagonist phentolamine were employed as specificity controls since these agents would not be expected to produce any effect.

Next, possible quenching or enhancement of the fluorescence of both DPH and 12-AS by these histamine and adrenergic drugs was tested. Again, no significant alteration in fluorescence intensity was detected for the H_2 and β agents or for the corresponding controls (H_1 and α agents).

Any effect on fluorescence intensity and maxima would have to be due either to a nonspecific interaction with the membrane (such as anaesthetic like properties observed with propranolol and antihistamines at high concentrations) or an indirect effect on the surrounding lipids (as a result of drug-receptor interaction affecting receptor-lipid interactions). It is possible that the use of fluorophores which reside preferentially at the polar head group region of the lipid bilayer or that associate themselves with the receptor proteins would pick up some differences here, although the possibility of nonspecific quenching due to the amino groups of all these drugs is quite likely.

4.2.2 Fluorescence Polarization

If receptor properties are substantially altered as a function of temperature, it may be a result of a change in the fluidity of the

surrounding lipids and/or in receptor-lipid interactions (Cook et al., 1977; Postel-Vinax et al., 1974; Radda, 1975b, c). Similar suggestions have been employed to explain discontinuities in Arrhenius plots for many membrane bound enzymes such as Mg^{2+}-ATPase, NADH oxidase, succinate oxidase, $(Na^{+} + K^{+})$-ATPase, and sarcoplasmic reticulum ATPase (Charnock and Bashford, 1975; Lenaz, 1979; Raison and McMurchie, 1974; Wunderlich et al., 1975). In many cases, a corresponding discontinuity in "fluidity" (either mobility or order) has been detected using DSC, NMR, ESR and fluorescence (Charnock and Bashford, 1975; Davis et al., 1976; Kimelberg and Papaholjopoulos, 1974; Lenaz, 1979; Raison and McMurchie, 1974). In fact, the technique of fluorescence polarization has been used to show such correlations. Examples include $(Na^{+} + K^{+})$-ATPase (Charnock and Bashford, 1975), two chromaffin granule membrane enzymes, Mg^{2+}-ATPase and NADH oxidase (Radda, 1975b), cytochrome P-450 oxygenase system (Narisimhulu, 1977), and thyroid-stimulating hormone receptor (Bashford et al., 1975).

The temperature dependence of 12-AS and DPH fluorescence polarization was, therefore, ascertained for the F3 fraction. For both probes, irrespective of the excitation wavelength, a single straight line could be drawn through the points; that is, no thermal discontinuity was observed with either probe in the range 44°C to 10°C. Thus, these results can be interpreted as meaning that no phase transition nor phase separation nor sudden change in fluidity occurs in this system. Another possibility is that if any sudden thermal change did occur, it was localized to a very small area of the lipid and was not able to be detected by

this method.

Drug-induced changes in fluorescence polarization have been observed. Propranolol was found to decrease the polarization of DPH in human erythrocyte membranes; that is, propranolol increased the membrane fluidity. This effect was suggested to a result of anaesthetic properties (Akiyama and Igisu, 1979). Insulin binding to its receptors was found to increase the overall microviscosity by about 10 to 20% in liver plasma membrane (Luly and Shinitzky, 1979). This was examined over the temperature range of 3°C to 40°C and the slope of the curve (described by a straight line) appeared to be slightly steeper in the presence of insulin, changing the apparent flow activation energy from 7.7 to 8.0 kcal/mole.

These findings led us to examine possible adrenergic and histaminergic induced alterations in the microviscosity of F3, as measured by fluorescence polarization, p. The temperature was varied from 40°C to 10°C in case receptor properties changed and thus the effects of the drugs on p appeared only within certain temperature ranges. These studies indicated that there was no significant difference in microviscosity, as measured by p for either DPH or 12-AS, upon addition of any of the drugs tested. Neither histamine and the H_2 antagonists cimetidine and metiamide nor the β-agonists INA and NA and the β-antagonist propranolol caused any alteration in polarization values of either probe in F3. The corresponding controls, that is, the H_1 antagonists, the α-agonist phenylphrine and the α-antagonist phentolamine, also had no effect. Even lowering the temperature did not alter their polarization

values significantly from those of F3 membranes untreated with any drug.

It, therefore, appears that interaction of the histamine and adrenergic agents with their receptors does not affect membrane microviscosity. This means that thermal alterations in drug receptor properties of either the histamine H_2 receptor or the β-adrenergic receptor in guinea pig cardiac sarcolemma do not exist or can not be detected using fluorescence polarization.

It is possible that thermal changes in the histamine and β-receptors do exist in guinea pig cardiac sarcolemma but can not be observed here. One possible reason is that the purification of the two receptors is insufficient for any effect to be measured. Further purification by removal of contaminating membranes might alleviate this problem. In a number of cases (Charnock and Bashford, 1975; Wilschut and Scherphof, 1974) prior to demonstration of thermal discontinuities in membrane fluidity, removal of some lipid (delipidation) is performed on the particular system in order that role of lipid surrounding the enzyme or receptor may be more effectively examined. It may be that these sudden changes in the lipid fluidity occur only in the "boundary" or "annular" lipids. This delipidation allows a greater portion of the observed parameter to be a result of these boundary lipids. One possible drawback is that extensive delipidation may produce an artificial milieu for the particular enzyme or receptor and thus any effects observed would be the result of this technique rather than a measurement of normal function. Proper monitoring of enzymatic activity and receptor

binding should accompany delipidation.

The use of site specific probes might allow better monitoring of receptor properties and lipid-receptor interactions. An example of such a probe is one which binds to the receptor protein (ideally by binding only to the particular receptor protein in question) covalently yet does not alter hormone binding or hormone induced responsiveness. Dansyl derivatives are available as fluorescent probes which covalently bound to proteins but their binding is not specific to any one protein. Receptor-specific fluorescent ligands would be of great advantage in studying receptor conformational changes and receptor-lipid interactions as a result of hormone binding.

4.3 Tissue Studies

In order to ensure that the conditions used did not inactivate the pharmacological receptors under study, the probes and their solvents were examined for their pharmacological effects on intact ventricular tissue. Neither DPH, its solvent THF, 12-AS, nor its solvent methanol had any significant effect on the histamine cummulative dose response curve (Figures 3.35 and 3.36). THF and methanol gave similar negative results on the INA cummulative dose response curve (Figures 3.37 and 3.38). DPH in the presence of THF may depress the INA curve although the results are not significantly different from controls. The large tissue variability in sensitization obscures interpretation of these data. 12-AS in the presence of THF may, on the other hand, increase the maximum response; however, the results are extremely variable and are subsequently not significant. The large tissue variability in initial responsiveness and subsequent sensitization with time requires the number of experiments to be increased so that true effects or lack of effects of DPH and 12-AS can be ascertained.

4.4 Summary

The results described in this thesis can be summarized as follows:

1. Partial purification of cardiac sarcolemma from guinea pig was achieved by successive differential centrifugation and sucrose gradient centriguation and without the use of perturbing high salt concentrations.
2. The presence of histamine H_2 and β-adrenergic receptors, as detected by agonist stimulation of adenylate cyclase, was observed in the cardiac sarcolemma fraction.
3. Contamination of this fraction by sarcoplasmic reticulum and outer membrane of the mitochondrion was difficult to assess due to the lack of proper marker enzymes. On the basis of those enzymes employed in this study to indicate the two membra tems, the fraction appeared to be contaminated.
4. Drug-receptor interaction could not be monitored in this system by using fluorescence intensity, excitation and emission wavelength maxima and fluorescence polarization of the lipophilic probes, DPH and 12-AS. It is possible, however, that fluorescent probes which are either located near the bilayer-aqueous interface or are covalently bound to the particular receptors in question could detect this interaction.
5. A continuous increase in fluorescence polarization of DPH and 12-AS in this system was obtained as the temperature was decreased from 44°C to 10°C. Thus, no sudden membrane fluidity change was detected

and could not, therefore, be responsible for any sudden change in cardiac adrenergic or histamine receptors.

6. The observations by some laboratories that α, β-adrenoceptor interconversion in heart tissue occurs is controversial. Studies dealing with the partial thermal transition of histaminergic receptors in guinea pig ileum in our laboratory have not been challenged. In general, sudden changes in membrane fluidity have been suggested as an explanation for receptor interconversion. Fluorescence studies presented in this thesis do not lend support to this suggestion either in the case of α, β-adrenoceptor or partial histamineric interconversion. However, if alterations in membrane lipids fluidity do occur, it may be small and localized to the annular or boundary lipids. To examine this possibility, the use of probes which preferentially located in these lipids is necessary.

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