FLUORESCENCE STUDIES OF PROLACTIN IODINATION AND PROLACTIN BINDING TO PITUITARY TUMOR CELLS

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ABSTRACT

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The study of plasma membrane lactogenic hormone receptors has been done predominantly via the use of ^{125}I-labelled hromones and partially purified membrane fractions. The kinetic and affinity constants derived from this radioreceptor assay (RRA) depend on the questionable assumption that the labelled and unlabelled hormones possess identical biochemical properties. A few investigators using unlabelled hormones and cell fractions have employed colorimetric analyses to describe hormone binding.

In this study, the lactoperoxidase catalyzed iodination of ovine prolactin was monitored with a computer coupled spectrophotometric-spectrofluorometer. A 34.7% decrease in tryptophanyl fluorescence and a 35.1% decrease in partial quantum efficiency were shown to correlate with the enzyme-dependent iodination reaction. The reaction mechanism appears to involve a tertiary complex of prolactin, lactoperoxidase and iodide. All fluorometric changes are complete within 5 minutes of the addition of hydrogen peroxide. The fluorescence response is clearly distinguishable from heavy ion collisional quenching and thus is believed to be the

result of stearic interference or conformational changes in the vicinity of the fluorophore.

The binding of ovine prolactin to a clonal strain of rat anterior pituitary tumor cells was also investigated by fluorescence techniques. As a criterion for metabolic activity, at least 90% of the intact cells were required to exclude trypan blue before and after the experimental reactions. The reaction is complete within 12 minutes and is characterized by dose-dependent tryptophanyl fluorescence enhancement. A sigmoid dose-response curve we interpret as showing potentiation of the binding site at concentrations between 18 and 30 μg/ml ovine-prolactin. There was no fluorescence enhancement in response to 25 μg/ml ovine growth hormone, but 45 μg/ml caused a small but significant degree of quenching. No hormone dependent changes in Tyndall or Rayleigh light scattering were detectable under the conditions of these experiments, however, methodological refinements are recommended for future investigations.

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A THESIS

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This thesis is dedicated to my mother

whose strength and courage are

a continuing inspiration.

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INTRODUCTION

The mammalian cell membrane is the structural and functional interface between the cell's environment and its cytoplasm. Electrical and chemical communication between the specialized tissues of the body are mediated and integrated by specific components of the plasma membrane of a cell. The exact compliment of proteins and lipids which compose the cell membrane is determined by the functional role that the cell plays and the physiological state of the animal. The plasmalemma also plays an important role as a transducer by converting electrical or bond energy into chemical signals which influence cytoplasmic processes such as protein synthesis, enzyme activation or nuclear transcription.

Polypeptide hormone receptors comprise a subpopulation of membrane proteins which are capable of stimulating precise and genetically prescribed cytoplasmic processes when triggered by the binding of the appropriate hormone. At this time, little is known about the mechanism of hormone binding and the majority of the work in this field has depended on the use of ^{125}I-labelled hormones. Using this radioreceptor assay, investigators have found that the binding is specific and saturable and the association is assumed to follow first order equilibrium kinetics. Any attempts to derive affinity constants rely on indirect analyses such as the Scatchard plot. A serious deficiency of the radioreceptor assay is the

assumption that the labelled and unlabelled hormone possess the same biochemical properties. There is no means of evaluating the validity of this assumption within this system because the only measurable parameter is the radioactivity itself. In order to avoid this dilemma, a few investigators have turned to colorimetric methods and unlabelled hormones to monitor the binding of the hormone to its receptor.

In recent years, prolactin and its receptor have been intensely investigated by a number of groups. The methods however have been limited to the use of I-labelled lactogens. Although the iodinated molecule is known to have a somewhat reduced biological potency, little effort has been spent in characterizing the molecular changes which account for the diminished activity. Because biochemical properties such as binding affinity and reaction rate constants have been derived for the iodinated species, it is essential to determine the applicability of these parameters to the native hormone. It would also be informative to develop a different assay method which could be compared to the radioreceptor assay.

This research was designed to examine the kinetics, mechanism and effects of the lactoperoxidase iodination reaction and compare the iodinated and native molecule by fluorescence methods. We also wished to explore the possibility of developing a fluorescence monitor of prolactin-receptor binding with intact cells.

LITERATURE REVIEW

I. Physical-Chemical Properties of Prolactin and Prolactin Iodination

A. The Prolactin Molecule

Prolactin is a polypeptide hormone secreted by the acidophil cells of the anterior pituitary in a variety of mammalian and sub-mammalian species. Purified monomeric prolactin, from the pituitaries of sheep (o-PRL), was first characterized by a group headed by C.H. Li (Li et al., 1969; Li et al., 1970). They found the hormone to consist of a single chain of 198 amino acids with no sugar or lipid moieties and having a molecular weight of 22,550. Prolactin's secondary structure is conferred by three disulfide bonds between cystines 4 and 11, 58 and 173, and 190 and 198. The molecule's isoelectric point is at pH 5.73. More recently, a number of lactogenic hormones have been isolated and characterized, including human growth hormone (h-GH), human chorionic sommato-mammotropin (HCS) (Li, 1972), bovine prolactin (b-PRL) (Wallis, 1974) and porcine prolactine (p-PRL) (Bewley and Li, 1975). Each of these hormones have a number of regions of amino acid homology and Li (1972) has used circular dichroism and optical rotary dispersion to show that each lactogen consists of 45 to 55 percent α-helix, and less than 12 percent β-sheet conformation. The distribution of charged and uncharged residues appears to be random in all the lactogens, but ovine, bovine, and procine prolactins are distinguished by having

only two tryptophan residues (Bewley and Li, 1975). Recently, Dombroske and Frantz (1976) have shown in vitro that rat prolactin may exist in monomeric, dimeric, or trimeric forms and the relative abundance of each species may be related to the rate at which they are secreted from the pituitary.

The structural-functional similarity of the various lactogens may be evaluated by comparing each hormone's capacity to stimulate a specific biological response. Accordingly, Niall (1972) finds that human prolactin (h-PRL), h-GH, and o-PRL all stimulate comparable epithelial growth of the pigeon crop sac. These findings were confirmed by Posner (1974), whose radioreceptor work shows that a number of lactogens, including rat prolactin (r-PRL) are all competitive for the same membrane receptor, but not all to the same degree. The biologically active region of the prolactin molecule has not yet been identified. However, Kawauchi (1973) has modified the biological activity of ovine prolactin by forming o-nitrophenylsulfenyl (NPS) derivatives of the native molecule. If a single NPS molecule is covalently bound to tryptophan-149, 80% of prolactin's biological activity is retained. In contrast, if NPS is bound to both tryptophan-90 and tryptophan-149, the resulting prolactin derivative is devoid of biological potency. These experiments suggest that the region surrounding trp-90 is essential to the biological activity of the hormone. Unfortunately, the tertiary folding of the prolactin amino acid sequence is not known,[1] and little information concerning prolactin's active site can be obtained from Kawauchi's experiment.

[1]Alexander Tulinski is presently attempting X-ray crystallography of o-PRL.

B. Lactoperoxidase Catalyzed Iodination of Prolactin

Radioiodination of polypeptide hormones, to high specific activity, can be accomplished by using chloramine T to oxidize the iodide (Greenwood et al., 1963). However, this reaction appears to destroy a large percentage of the hormone's biological activity. To alleviate this problem, Frantz and Turkington (1972) developed an enzymatic method of prolactin iodination using lactoperoxidase from cow's milk to catalyze the reaction. Low concentrations of peroxide are provided to energize the reaction. Similar methods have also been used by others for other polypeptide hormones (Thorell and Johansson, 1971; Posner, 1974) and all report high specific activity (about 130 μCi/μg) and about 60% biological activity. The pH maxima of lactoperoxidase are at 5.6 and 7.4; the former being nearly twice as great as the latter (Rogol and Chrombach, 1975).

In spite of the widespread use of the lactoperoxidase iodination method, little is known about the mechanism of the reaction for large polypeptides. The reaction involves three substrates: peroxide, iodide and the phenolic compound which is iodinated. Morrison and Bayse (1970) have studied the kinetics of tyrosine iodination and report that a quaternary complex is not formed, but that a "ping-pong-type" mechanism is involved. They caution however, that their proposed mechanism may not be applicable to protein substrates. In fact, there is no final evidence which implicated tyrosine as opposed to phenyalanine or tryptophan as a site of iodide fixation in polypeptides. However, lactoperoxidase has been used to iodinate the

tyrosine side chains of thyroglobulin (Monoco et al., 1975), but because these tyrosines are not in peptide linkage, we cannot conclude that tyrosine is a preferred substrate for enzymatic reaction with proteins. Moreover, there is some evidence that lactoperoxidase may also catalyze the oxidation of indole compounds such as tryptophan. This indole oxidation (Alexander, 1974) may partially account for the reduced biological activity of radioiodinated prolactin.

II. Prolactin Synthesis and Secretion

Both prolactin and growth hormone are synthesized and secreted by the acidophil cells of the anterior pituitary (Li, 1969). A specific messenger RNA has been isolated and used to synthesize prolactin in cell free systems (Maurer, 1976; Evans and Rosenfeld, 1976). The regulation of prolactin release is under the control of a number of inhibiting and stimulating factors. Meites (1959) found that prolactin secretion was stimulated by surgically separating the pituitary from the hypothalmus, and Talwalker (1963) was able to inhibit prolactin secretion in vitro by exposing pituitary explants to hypothalamic extracts. Both of these phenomena illustrate the effects of a prolactin inhibiting factor (PIF). A prolactin releasing factor was first proposed by Meites (1961), who was able to stimulate lactation by injecting a crude hypothalamic extract into estrogen primed rats. It soon became evident however, that other humoral factors were effecting the secretion of prolactin. For example, Yamamoto (1970) injected ^{14}C-leucine into male and female newborn rats and followed the secretion of prolactin during the course of the rat's development. Between days 37 and 45, the female rats

showed a rapid increase in the concentration of labelled serum prolactin. The males also had increased serum prolactin levels by day 45, but in all cases the radioactivity per hundred grams body weight was higher for females than males. It was also found that the pituitary content and plasma concentrations of prolactin in female rats, are higher during estrus and proestrus as compared to diestrus (Ieiri, 1971). More recently, Meites (1972) has reported that estrogen can stimulate prolactin secretion not only via the hypothalamus, but directly at the pituitary. The action of progesterone as an effector of prolactin secretion is not clear, but Blake (1972) concludes that the roles of estrogen and progesterone in prolactin secretion are probably dose and system dependent.

Thyrotropin releasing factor, the catecholamines and the biogenic amines also appear to moderate the rate of prolactin secretion. Shaar and Clemens (1974) report that 2.5 to 5 μg/ml of dopamine are sufficient to significantly inhibit prolactin secretion in vitro, while equivalent doses of catecholamines have no apparent effect. On the contrary, Chen and Meites (1975) found that both catecholamines and dopamine precursors are able to stimulate the release of prolactin inhibiting factor, and thus effect a decrease in serum prolactin titers. However, it may be that pharmacological doses of catecholamines are necessary to be effective. Serotonin and serotonergic precursors by contrast tend to stimulate prolactin secretion (Kamberi et al., 1971). These findings have led to the suggestion that the biogenic amines control diurnal fluctuations in prolactin serum levels (Meites et al., 1972). Tashjian (1971) has

shown that the tripeptide, thyrotropin releasing hormone (TRF), is capable of stimulating the secretion of prolactin from cultured pituitary tumor cells when administered at 0.1-10 μg/ml concentrations. He also notes that TRH increases the rate of prolactin synthesis while the rate of degradation remains constant. In addition, 5×10^{-6} M hydrocortisone was sufficient to inhibit the release of prolactin (Dannies and Tashjian, 1973). In vivo stimulation of prolactin secretion in the rat by TRF was demonstrated by Rivier and Vale (1974) who were also able to quantitate the various effects of TRF analogs. The rye ergot derivatives such as CB-154 are also able to reduce serum prolactin levels in rats as was reported by Brooks and Welsch (1974). The mechanism of their action is probably via increased PIF from the hypothalamus.

There are conflicting reports concerning the effect of prolactin on its own secretion. Voogt and Meites (1971) have shown that a 250 μg implant of prolactin in the median eminence of pseudopregnant rats inhibits the secretion of prolactin probably by stimulating dopaminergic neurons in the hypothalamus (Fuxe and Hokfelt, 1970). In addition, the prolactin implant caused leuteinizing hormone and follicle stimulating hormone titers to increase, and the rats resumed their estrus cycles. Macleod and Abad (1968) on the other hand, observed direct inhibition of prolactin secretion in rats with multiple pituitary tumor explants. It is interesting to note that Frantz's group (1975) has found specific radioreceptor binding sites for ^{125}I-prolactin in cultured anterior pituitary tumor and normal cells; and Payne (1975) correlated the binding

capacity of these cells with the rate at which they secrete prolactin. There is no final conclusion nontheless, and the mechanism of the short-loop feedback system awaits more detailed investigation.

III. Radioreceptor Assays for Prolactin Binding

Birkinshaw and Falconer (1972) injected ^{125}I-labelled ovine prolactin into female rabbits and followed the tissue distribution of radioactivity by autoradiographic techniques. Their report showed that the radioactivity appeared to be bound to the plasma membrane of the alveolar secretory cells of the mammary gland. This study prompted the development of a radioreceptor assay which proved to be a sensitive and specific method of detecting prolactin binding to target tissues. The properties of the lactogenic receptor were first described by Frantz (1972) who showed that the binding sites are protease sensitive, responsive to nanogram concentrations of hormone, and distributed to a number of tissues including mammary, adrenals, liver, prostate and seminal vesicles. The kinetics of the binding reaction are still not clearly understood. Frantz reports saturation times of about thirty minutes and Shiu and Friesen (1974) report saturation times of up to three hours.

The functional significance of prolactin binding has been established by a number of physiological events including casein synthesis and α-aminoisobutyric acid (AIB) transport in mammary tissue (Frantz et al., 1973; Shiu and Friesen, 1976). Thus, the prolactin receptor is consistent with the structural-functional criteria set forth by Cuatrecasas (1974). More recently, prolactin receptors have been found in the pituitary (Frantz et al., 1975),

and in estrogen receptor-deficient mammary carcinomas (Costlow et al., 1973). Moreover, Kelly's group (1974) has shown that specific binding of prolactin to mammary tumors correlates strongly with the dependence of the tumor on prolactin.

The relative abundance of prolactin receptors in rat liver correlates to sex, pregnancy, and state of lactation. This observation led several investigators to explore the effects of sex steroids, hypophysectomy and prolactin itself on the regulation of liver receptors. Kelly et al. (1975) noticed that in female rats, receptor induction occurs during estrus and diestrus I, while receptor reduction occurs during proestrus and diestrus II. Similarly, estrogen injections can augment prolactin binding in males. These effects however, take up to six days to be manifested, while hypophysectomy has a comparable effect within forty-eight hours (Posner et al., 1974). When pituitary glands are implanted in the renal capsule of hypophysectomized female rats, a rapid rise in serum prolactin titers ensues which is paralleled by receptor induction in the liver (Posner et al., 1975). These effects can be duplicated by a single 2 mg injection of ovine prolactin, and the results are independent of serum steroid levels (Costlow et al., 1975). Thus, prolactin itself is at least partially responsible for the induction of its own receptor. Shiu and Friesen (1974) have solubilized and purified the membrane receptors from rabbit mammary tissue and found that it is proteinaceous in nature. Moreover, a guinea pig antiserum to prolactin receptors inhibits not

only prolactin binding, but the ability of the hormone to stimulate casein synthesis and AIB transport in mammary explants (Shiu and Friesen, 1976).

In spite of the apparent utility of the radioreceptor assay, a number of discrepancies are seen when it is compared to biological assays. The ultimate reliability of the system has not yet been proven, and a number of criticisms have been advanced by Nicoll (1975). In particular he finds that prolactin binding does not always correlate with the initiation of a biological response.

IV. Pituitary Cell Culture as a Model for Organ Function

Long term culture of mammalian pituicytes is limited to the use of tumor cells because normal cells have a tendency to de-differentiate or die after several weeks in culture. Several anterior pituitary cell lines were isolated and characterized from X-ray irradiated Wister-Furth rats by Tashjian (1968). He found that three of these clonal strains secreted both growth hormone and prolactin and were stable in culture over extended periods of time (Tashjian et al., 1970). The karyotype, specific function, and appearance of the cells were also documented (Sonnenschein et al., 1970). Following this extensive characterization of the cell lines, a specific functional criterion was examined: the effect of thyroid releasing factor (TRF) on the secretion of prolactin. They found that the rate of prolactin secretion by one strain of cells was quantitatively related to the amount of TRF provided in the growth medium, while a second strain of cells was relatively unresponsive

to TRF (Tashjian, 1971; Dannies and Tashjian, 1973). These biological responses were subsequently correlated with specific binding of radiolabelled TRF to a membrane receptor (Tashjian, 1973). The receptor affinities and biological potency of TRF analogs were also studied and the active site of the hormone molecule was postulated (Hinkle, 1974). Finally, differential fluorometry was used to monitor the conformational changes of membrane proteins which accompany the binding of TRF to cell homogenate particles (Imae et al., 1975).

A second group of cell lines cloned from estrogen induced rat anterior pituitary tumors was characterized by Sonnenschein (1973). The morphology, growth properties, and secretory products of the cells have also been described (Sonnenschein et al., 1974). The authors caution however, that morphology does not correlate with function, and both histologically different cell types secrete prolactin and growth hormone. Three of Sonnenschein's clonal cell strains were assayed for prolactin binding. One was found to have slightly greater binding capacity than normal pituitary cells, while the other two strains were devoid of prolactin receptors (Frantz et al., 1975). The physiological significance of these binding sites is still undetermined.

Although pituitary tumor cells appear to be excellent models for endocrinological investigation, there are important physiological properties which distinguish these cells from normal cells. First of all, tumor cells generally have lost their density dependent regulatory apparatus (no contact inhibition) and thus will continue to multiply indefinitely (Abercrombie, 1970). Franks (1968) has

determined that tumor cells often possess surface antigens which distinguish them from normal cells. In addition, the carbohydrate content of the surface proteins of tumor cells appears to be altered. The latter property permits tumor cells to be selectively agglutinated by plant lectins such as Concanavalin A and wheat germ agglutinin (Cook and Stoddart, 1973). All of these characteristics suggest that tumor cells have unusual and distinctive membrane properties which may be significantly different from normal cells. Therefore, any conclusions drawn from *in vitro* experiments must be cautiously and prudently generalized to *in vivo* conditions.

V. Structure and Physical Properties of Cell Surface Membranes

A. Overview

The plasma membrane of the eukaryotic cell is the only structural and functional barrier between cytoplasmic processes and the cell's environment. Thus, all cellular nutrients and waste products must either diffuse or be carried across this lipo-protein partition; and electrical or chemical stimuli must be communicated to the cytoplasm by membrane-mediated phenomena. In the present study, the hormone-membrane interactions of prolactin and its membrane binding site have been investigated, and a bried review of the literature concerning the structural-functional properties of membranes is appropriate. The classical article by Danelli and Davson (1935) gave us the first insights into the conformational arrangements of lipids and proteins into a bimolecular sheet which was believed to be essentially rigid and inelastic. The concept of a rigid membrane

was also supported by electron microscopic and X-ray diffraction studies which presented the membrane as an orderly array of lipids and imbedded proteins (Hendler, 1971). However, during the past fifteen years, a number of biochemical and biophysical techniques have led us to believe that the components of the plasma membrane are not static, but undergo numerous conformational and configurational changes. A few of the pertinent techniques and results which have furthered this dynamic concept of the plasma membrane are presented below.

B. Infrared Spectroscopy

Interatomic distances within molecules fluctuate about average values through one or more vibrational motions. Such motions can change the dipole moment of a given bond and the resulting electric field will oscillate at the same frequency as the bond vibrations. If the bond is irradiated with electromagnetic waves of the same frequency, it absorbs some of the radiant energy in a quantal fashion. The absorption spectra of various chemical bonds have been catalogued and serve as a reference for the interpretation of macromolecular microenvironments. Thus, infrared (IR) absorbance spectra are useful for determining the interactions of macromolecular components of membranes. For example, Maddy and Malcolm (1965) have determined that the phosphate ester stretching (P=O) frequency in erythrocyte ghosts is the same as that observed in phospholipids in water. This suggests that the polar heads of membrane lipids are oriented toward an aqueous

environment. Wallach and Zahler (1968) compared the IR spectra of Ehrlich ascite tumor cell membranes with the IR spectra of free lipids and concluded that 25% of the lipid must be hydrophobically bound to membrane proteins. Moreover, the IR spectra of erythrocyte membranes indicate that the imbedded proteins are capable of conformational changes which correlate with metabolic processes such as ATPase activity. There is also evidence that membrane proteins may possess a substantial amount of β structured H-bonding (Chapman, 1973).

C. Nuclear Magnetic Resonance (NMR)

The nuclei of many atoms are like spinning charged spheres. These oscillating electric fields induce localized magnetic moments which can be oriented in an applied field. If electromagnetic radiation is applied, the nucleus may be forced to realign itself. NMR essentially measures the energy required for the realignment, and thus supplies information about neighboring molecules and their configurations (Wallach and Winzler, 1974). Of particular interest in the study of biological membranes is proton magnetic resonance (PMR), which in general is thought to arise primarily from the membrane lipids. There is a distinct difference in NMR and PMR spectra of erythrocyte ghosts and sonicated membranes. The sonicates tend to have sharper, well defined peaks and longer relaxation times (Chapman et al., 1968). The broad and indistinct peaks found in the membrane ghosts implies a more structured and thermodynamically stable arrangement of molecules. Conversely, excitable membranes

such as the sciatic nerve of the rabbit, appear to have regions of relative fluidity (Dea et al., 1973). Glaser (1970) has studied the effects of temperature on PMR and reports that an increase from 18 to 40°C will greatly enhance the liquid content of the membrane lipids. Only at temperatures above 60°C will the methyl groups from proteins contribute to the spectra. In addition, they found that phospholipase C can increase the fluid nature of erythrocyte membranes by 75%, without changing the tertiary structure of the membrane proteins. They concluded that the proteins and lipids interact in a "fluid mosaic" medium.

D. Electron Spin Resonance (ESR)

The spinning charge of an electron will induce a magnetic field. Generally, electrons are paired in chemical bonds and their magnetic moments cancel. In contrast, molecules such as nitroxides contain linkages with unpaired electrons. When placed in a magnetic field, the unpaired electrons orient themselves either with or against the field. If electromagnetic radiation, of appropriate frequency is applied, energy may be absorbed or emitted and the electron can "flip" its alignment. A difference in the relative populations of the two alignment states is the basis of electron spin resonance spectroscopy. Because most biological molecules do not possess unpaired electrons, covalently bound nitroxide labels must be affixed to the molecule being studied. Kornberg and McConnell (1971) used spin labelled ESR to monitor the inside-outside exchange of lipids between the bimolecular layers of phosphatidylcholine

micelles. They estimated the "flip-flop" half times to be about 6.5 hours at 30°C. On the other hand, lateral diffusion of lipids in the plane of the membrane is relatively rapid. Thus, neighboring lipids can exchange places with a frequency on the order of 10^7 per second (Devaux and McConnell, 1972). However, this lateral diffusion rate is apparently proportional to the lipid/protein ratio in the membrane. Jost (1973) found that as the concentration of cytochrome oxidase in a model membrane was increased, the fluidity of the lipids decreased. They determined that about 0.2 mg of phospholipid is hydrophobically bound to each mg of protein. It should be noted that some researchers believe that the spin-label itself may contribute to the fluidity of the membrane. Recently, this criticism has been avoided by Stanacev and Stuhne-Sekalec (1974) who have perfected an enzymatic incorporation of radio-labelled and spin-labelled phosphatidic acid. The radiolabel (^3H) permits easy quantitation and the spin-label is biologically active stearic acid.

E. Fluorescence Spectroscopy

Electrons revolving in their respective orbits are capable of absorbing electromagnetic energy in a quantal fashion. The fate of the absorbed energy is a function of the molecular environment of the chromophore. Ideally, an electron can emit radiation one of two ways: emit a photon from the same vibrational level to which it was excited, or undergo changes in vibrational levels prior to emission of radiation. In solution, only the latter phenomenon is observed and the emitted radiation (fluorescence) is always of longer wavelength (Hercules, 1966). Proteins are complex molecules and usually

contain more than one chromophore and fluorophore. Under favorable conditions an excited residue may transfer its energy to an absorbing residue which in turn will emit photons at its characteristic wavelength. The efficiency of the energy transfer varies with the inverse sixth power of the separation of residues so that generally a donor-receptor pair must be within 20-100 Å of each other for appreciable energy transfer to be detected (Van Holde, 1971). X-ray diffraction analyses have shown that such distances are common between the aromatic residues of biological proteins (Tulinsky et al., 1973; Liljan et al., 1972). Because of this energy transfer and the fact that the tryptophan absorption band is at the longest wavelength, it is not surprising that when phenylalanine, tyrosine and tryptophan are all present in a protein, tryptophan fluorescence usually constitutes the major emission peak.

Fluorescence spectroscopy may be used as a sensitive monitor of many substrate-ligand binding reactions. The association of a protein with a substrate is often accompanied by fluorescence perturbation which can be stoichiometrically related to the concentrations of reactants. Examples of such reactions are the tubulin-colchicine system (Bhattacharya and Wolff, 1974) and lysozyme-saccharide binding (Halford, 1975). In addition, selective quenching of protein tryptophan fluorescence can be effected by exposing the protein to various concentrations of heavy ions such as iodide (Lehrer, 1967). If a mathematical analysis of tryptophan quenching is subsequently made, valuable information about protein tertiary structure can be resolved (Lehrer, 1971).

The conformational and structural arrangement of membrane proteins have been studied by a number of fluorescence techniques. In particular, 1-anilino-8-naphthalene sulfonate (ANS) and 2-p-toluedinylnaphthaline-6-sulfonate (TNS) have been employed extensively as membrane fluorescent probes. The major factors effecting ANS and TNS fluorescence are viscosity, polarity, and polarizability of their microenvironment (Oster and Mishyma, 1956). For example, the transfer of a naphthaline sulfonate derivative from an aqueous to an organic solvent will cause a large enhancement of fluorescence and an accompanying blue shift, i.e., fluorescence at a lower wavelength. There is also energy transfer from membrane tryptophans to membrane bound ANS. This is apparent both from the excitation spectra of free and membrane bound ANS, and the quenching of tryptophan fluorescence by binding of ANS (Wallach, 1970). Gulik-Krzywichi (1970) has used X-ray diffraction and fluorescence to show that ANS is usually bound in the hydrophobic regions of protein-lipid contact. The utility of ANS as a membrane probe was admirably demonstrated by Tasaki (1968) and Teisse (1975) who have monitored fluorescent changes which parallel depolarization of isolated nerve axons. Feinstein (1970) has also reported fluorescence enhancement of membrane bound ANS during treatment of erythrocytes with butacaine and calcium. Each of these experiments suggest that membrane proteins undergo conformational changes which facilitate or inhibit the transfer of electromagnetic energy from tryptophan to ANS.

The fluorescence of membrane protein itself can be monitored when appropriate excitation and emission wavelengths are selected.

Membrane fragments from Erhlich ascite tumor cells emit light at 335 nm when excited at 275 nm which implies that membrane tryptophans are located in a somewhat non-polar environment (Wallach and Zahler, 1966). These membrane fragments also lacked an emission peak at 303 nm which suggests that energy transfer from tyrosine occurs. Similarly, Sonenberg et al. (1971) reports that human erythrocytes reacted with human growth hormone emit 20% less fluorescence than membranes alone. Concurrent with this apparent tryptophan quenching is an observed decrease in fluorescence polarization. The effect was restricted to pH 7.4 and physiological temperatures. When growth hormone is reacted with rat liver membranes a corresponding fluorescence quenching is observed and a greater negative ellipticity (monitored by circular dichroism) is found to correlate with 5'-nucleotidase activity (Postel-Vinay, 1974; Rubin, 1973). Imae et al. (1975) could also observe fluorescence quenching associated with treatment of cultured pituitary cell fractions with TRF. The quenching effect could be titrated with increasing quantities of hormone. One of the most revealing experiments concerning membrane structure and functions was conducted by Taylor (1971). His group reacted fluorescence-labelled anti-immunoglobulin with intact mouse spleen cells. They found that immediately following the reaction, the labelled antigen was scattered randomly over the surface of the cell. However, within 30 minutes, the fluorescing particles had migrated to one of the poles of the cell: a phenomenon called capping. These experiments suggest that proteins as well as lipids can be highly mobile in the plane of the membrane.

In light of the experiments outlined above, it is not surprising that models of cell membranes have undergone considerable revision during the past decade (Bretscher, 1973; Siekevitz, 1972). It is also quite reasonable to suspect that membrane reactions may have direct effects on cytoplasmic phenomena (Edleman, 1976).

MATERIALS AND METHODS

I. The Spectrophotometric-Spectrofluorometer

The instrument used for the majority of the present study is a spectrophotometric-spectrofluorometer (Holland et al., 1973) coupled to an on-line computer which applies corrections to many of the instrumental and photophysical variables of fluroescence measurements. The unique feature of this instrument is its capacity to measure absorbance and fluorescence simultaneously from paired quartz cuvettes. The contents of one cuvette serve as a reference, while the contents of the second cuvette are varied through one or several experimental parameters. The excitation radiation to each cuvette is derived from a single monochromatic light source which is chopped by an oscillating mirror, and passed intermittently (25.0 milliseconds) to each cell. The transmitted photons are then fed to a photodetector which in turn feeds the signal to the computer. The 90° emission from the experimental (or sample) cuvette is passed to a second monochrometer and photodetector and ultimately to the computer. Thus, a single excitation scan will provide the data needed to compute simultaneous absorbance and fluorescence spectra. Moreover, when the number of quanta fluoresced is divided by the number of quanta absorbed, a unique datum is derived. Since at any point along the scan axis, the emission detector will see only a fixed part of the total quanta fluoresced, this datum has been called

partial quantum efficiency (PQ). Ideally, changes in PQ linearly exhibit the changes in total quantum efficiency. Thus, chemical reactions in the sample cuvette which produce subtle changes in solvation, conformation, or bonding in the vicinity of the fluorophore may also produce changes in PQ. Therefore, like fluorescence, PQ presents an intrinsic quantity which can be used to detect structural changes affecting the relaxation processes of the photoexcited molecule. Because PQ is the quotient of the number of quanta fluoresced and the number of quanta absorbed, it is unitless, and thus, in the case of a pure fluorophore, independent of excitation wavelength and concentration. The term Relative Fluorescence Efficiency has also been used to define this quantity.

The computer which is coupled to the fluorometer is connected to a teletype which is equipped with a phosphorescent screen. The data collected during an excitation or emission scan can be displayed visually in tabular or graphic form. There are three programs which are employed extensively during the present study. (1) The excitation scan is simply the collection of absorbance, fluorescence and PQ values while the emission monochrometer is held at a constant setting and the excitation monochrometer is driven over a pre-determined range. (2) Similarly, the emission scan is the collection of data while the excitation wavelength is held constant and the emission monochrometer is varied. (3) In the time scan, both monochrometers are held at pre-determined settings and data is collected over discrete periods of time. In the present study, all time scans were performed during five second intervals when 12-15 data points

were collected for absorbance, fluorescence, and PQ. These data were averaged by the computer and displayed on the fluorescent screen of the teletype. Henceforth, the averaged time scan data shall be referred to as AB (absorbance), CO (corrected fluorescence), and PQ (partial quantum efficiency). With this instrument, the units of absorbance and fluorescence are relative and can be varied by adjusting the photomultiplier amplifier voltage and the monochrometer slit width settings. Throughout this study the instrument was calibrated with the same 10^{-5} M quinine sulfate solution in 0.1 M H_2SO_4.

A diagram and brief discussion of the spectrofluorometer is provided in Appendix A.

II. Reactions with Prolactin

A. Iodination

Preparations of ovine prolactin (NIH-P-S-11), unlabelled KI (Baker Chemical Co., Phillipsburg, N.J.), H_2O_2 (Mallinckrodt Chemical Works, St. Louis, Mo.) and lactoperoxidase (Calbiochem, San Diego, Ca.) are made to equal ten times the concentrations used by Frantz (1972). The prolactin and lactoperoxidase are weighed on a Cahn model 4100 electrobalance (Cahn Corp., Paramount, Ca.) and stored at -27°C until two hours before use. Experiments in our lab (unpublished) have shown that prior dissolution of prolactin in 0.01 M NH_3HCO_3 (pH 8.3) will facilitate the solvation of hormone in the buffer which provides the optimal pH for lactoperoxidase activity: 0.4 M sodium acetate, pH 5.3. Accordingly, 25 µg of KI, and 100

nanomoles of H_2O_2, directly to the prolactin solution. Each solute in 0.25 ml of sodium acetate (Baker Chemical Co.) buffer is added via a Hamilton microliter syringe. The reaction is monitored by the spectrophotometric-spectrofluorometer described in the previous section.

B. Potassium Iodide Quenching

The selective quenching of tryptophanyl fluorescence by KI has been demonstrated by Lehrer (1971) for a number of model polypeptides. In the present study, 25 μg/ml solutions of ovine prolactin (NIH-P-S-11) were prepared in 0.4 M Na Ac (pH 5.3) containing 0.00, 0.04, 0.12 or 0.2 M KI. To inhibit I_3 formation 10^{-4} M Na_2SO_3 was also provided. (All chemicals are analytical grade.) The relative fluorescence of each solution was determined with an Aminco-Bowman spectrofluorometer.

III. Pituitary Cell Culture

A. Growth Medium

$C_8$11RAP rat anterior pituitary tumor cells are raised in 3 liter roller bottles at 37°C essentially according to the method of Payne (1975). The growth medium is prepared using a modification of Sonnenschein's medium (1974), consisting of 13.47 g per L Dulbesco's Modified Eagle Medium Powder (GIBCO, Grand Island, N.Y.), 15% Horse Serum (Difco, Detroit, Mi.), 2.5% Fetal Calf Serum (Difco), 812 mls triple distilled water, and 0.5 m M N'-2-hydroxyethylpiperazine-N'-ethanesulfonic Acid (Hepes) buffer (GIBCO). An antibiotic-antimycotic mixture (GIBCO) and 72 μg of Anti-PPLO-agent (GIBCO)

are also added (a Tylocine preparation). The medium is pH adjusted to 7.2 with sodium bicarbonate (GIBCO). Before use, the medium is filtered through a 0.45 μ pore filter (Gelman Instrument Co., Ann Arbor, Mi.) utilizing a sterile pyrex Millipore-filtering apparatus (Millipore Corp., Bedford, Ma.). The medium is then frozen (-20°C) until needed.

B. Harvesting and Preparation of Cells

Cells are harvested and fed under a laminar flow hood (Type W S series 300, Westinghouse, Grand Rapids, Mi.). Within six hours of an experiment, cells are harvested and centrifuged at 1800 rpm's for 15 minutes in a Servall model 554 centrifuge (Sorvall Corp., Newton, Conn.). The culture medium is decanted and the pellet resuspended, washed three times, and concentrated in $Pucks_f$ plus glucose (a protein-free balanced salt solution, pH 7.4; see Appendix B). Aliquots of the resulting cell suspension are counted with a Neubaurer hemocytometer and tested for viability by excluding 0.01% trypan blue in 0.02 M citric acid (or 0.01% Erythrocin B). Tennant (1964) found 85% of the cells counted by this method were capable of replication under optimal conditions. In this study, 90% of the cells were required to exclude the dye both before and after the experimental reactions. The cells were viable in $Pucks_f$ plus glucose for at least six hours.

IV. Fluorescence and Light Scattering Monitors of AP Cells: Prolactin Reactions

A. Fluorescence

The reaction of a polypeptide hormone with erythrocyte membrane homogenates was first performed by Sonenberg (1969). He found that picomolar quantities of human growth hormone were sufficient to cause a twenty percent reduction in the intrinsic fluorescence of membrane tryptophans. Similarly, Imae (1975) found that TRF will effect comparable quenching of membrane fluorescence of cultured AP cell particles. In the present study, 87.5, 125, 150, 225, 250, and 500 µg/ml solutions of ovine prolactin (NIH-P-S-11) were prepared in $Pucks_f$ saline plus glucose. With a microliter syringe, 250 µl aliquots of those solutions were then injected into 2 mls of various concentrations of $C_8$11RAP pituicytes in the sample cuvette of the fluorometer. Thus, the resulting suspensions contained 17.5, 25, 30, 45, 50, and 100 µg/ml of hormone, respectively; the reference cuvette contained only $Pucks_f$. AB, CO, and PQ readings of the preinjection and postinjection suspensions were taken at various time intervals. The pituitary cells were prepared as described above, and suspended in $Pucks_f$ plus glucose. As a control, 87.5, 125, and 225 µg/ml solutions of ovine growth hormone (NIH-GH-S10) were also prepared and reacted with the cells as above. All reactions were conducted at 4°C in the water cooled fluorescence chamber of the spectrofluorometer.

B. Light Scattering

Individual $C_8$11RAP cells have a diameter on the order of 7 microns (Sonnenschien, 1973). Thus, they are capable of scattering a substantial portion of the light which is incident on them (Van Holde, 1971). In order to distinguish the light scattering effects from fluorescence effects it is appropriate to conduct light scattering experiments which parallel the fluorescence analysis experiments. We prepared various concentrations of $C_8$11RAP cells which were reacted with o-PRL at a final concentration of 40 µg/ml of hormone. The conditions of the experiments were identical to those used in fluorescence experiments except the excitation and emission monochrometers were both set at 330 nm wavelengths. As a control, comparable suspensions were injected with 250 µl aliquots of $Pucks_f$ plus glucose.

V. Microphotography

Photomicrographs of $C_8$11RAP suspensions were taken with a 35 mm Yashika TTL reflex camera (Yashika Co., Tokyo, Japan) using a Honeywell Pentax microscope adapter (Honeywell Corp., Denver, Colo.) and a Unitron model Mic 2312 inverted microscope. The film used was Kodak Tri-X (Eastman Kodak Co., Rochester, N.Y.).

EXPERIMENTAL

I. Iodination and Iodide Quenching

A. Iodination

1. Objectives

The radioiodination procedure of Frantz (1972) is known to yield some prolactin species which are deficient in biological activity as measured by the radioreceptor assay method. In this experiment, the iodination reaction is monitored by fluorescence instrumentation in order to derive information concerning the mechanism, kinetics, and site of iodide fixation.

2. Procedures

Solutions of 25 µg/ml of o-PRL were prepared two hours prior to iodination. Quadruplicate 2 ml aliquots of PRL in sodium acetate buffer were pipetted into the sample cuvette of the fluorometer. Five time scans were performed at two minute intervals on each sample. Then 100 µg of lactoperoxidase was injected into the cuvette in 0.25 ml of acetate buffer, followed by syringe agitation. The contents of the cuvette were allowed to equilibrate for about two minutes and then 3-5 time scans at 2 minute intervals were performed. Similarly, 0.26 µg KI was injected and followed by time scans. When 0.25 ml of the peroxide was added an immediate time scan was taken and 5-10 subsequent time scans at one minute intervals were performed. A final time scan was taken approximately 30 minutes

following the initial injection of peroxide. The protocol for the control experiments was identical to the above procedure except lactoperoxidase was not added to the cuvette. For all reported experiments, the excitation monochrometer was set at 288 nm and the emission monochrometer at 380 nm.

AB, CO, and PQ values for each time scan were recorded and cataloged according to the time course of the reaction and the addition of reactants, respectively. All other possible combinations of PRL, lactoperoxidase, KI, and H_2O_2 were also assayed for fluorometric responses according to the protocol outlined above. Of all the control experiments, only the PRL plus H_2O_2 and the lactoperoxidase deficient control elicited measurable responses; and both of these were significantly less than the complete reaction mixture response.

3. Results

a. Kinetics.--AB, CO, and PQ values obtained from the computer printout are expressed in arbitrary units which are a function of the excitation slit width and the voltage applied to the photomultiplier amplifier; both of which were set at constant levels. The raw data obtained from various stages of the reaction are also proportional to the concentration of reactants in the cuvette. Since each reactant is added in a 0.25 ml sodium acetate vehicle, a proportional dilution correction must be applied to the respective raw data. No dilution correction is necessary for PQ.

Figure 1 is a representative graph of dilution corrected AB, CO, and PQ versus time. The addition of each reactant is marked

Figure 1.--Absorbance, fluorescence and partial quantum efficiency changes during an iodination reaction plotted against time in minutes.

● = absorbance x 10.

o = fluorescence.

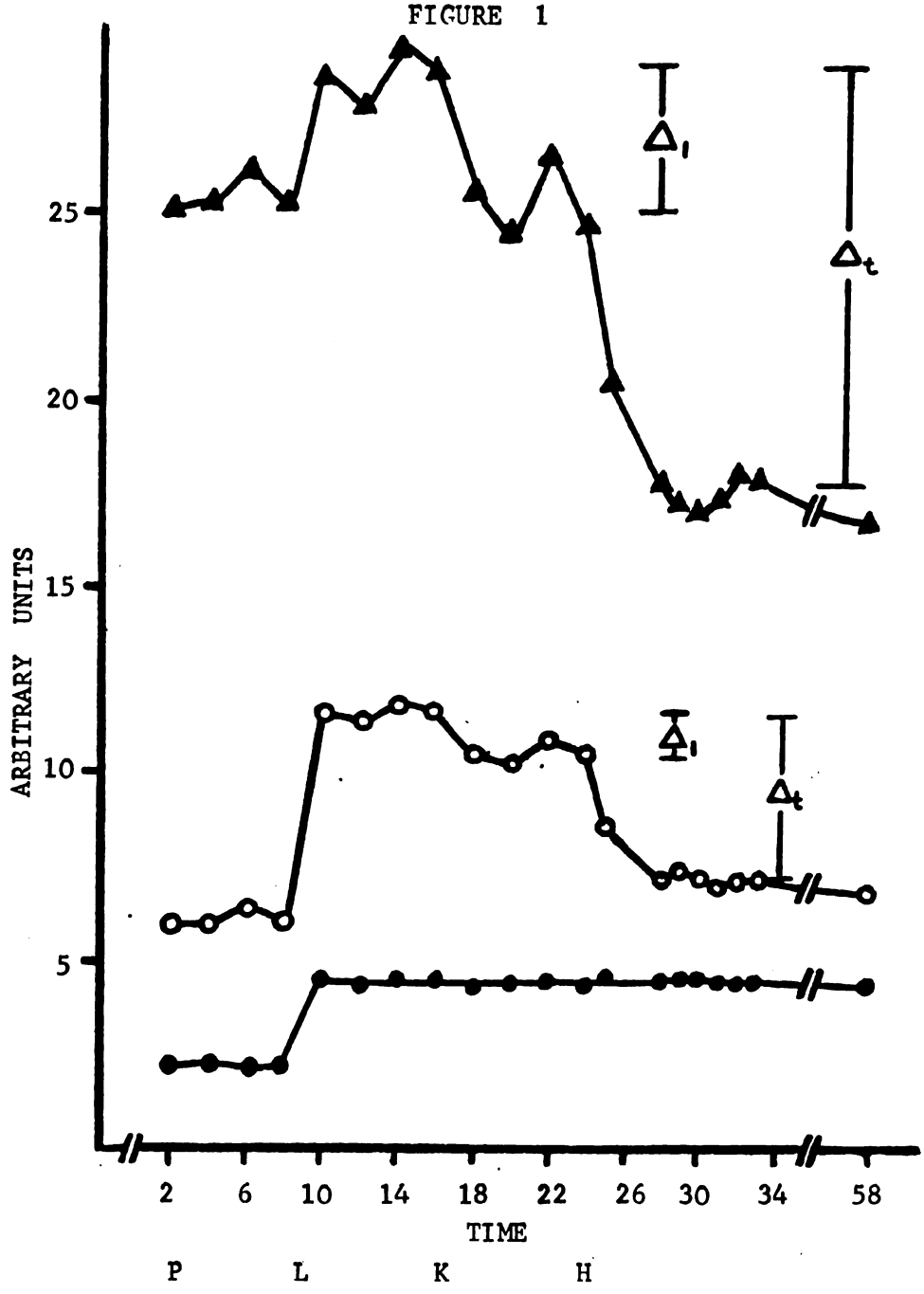
▲ = partial quantum efficiency.

Letters beneath the abscissa designate the addition of PRL(P) lactoperoxidase (L), KI(K), and H_2O_2(H).

Δ_1 = change in CO or PQ six minutes following the addition of KI.

Δ_t = change in CO or PQ eight minutes following the addition of H_2O_2.

FIGURE 1


ARBITRARY UNITS
25
20
15
10
5
Δ_i
Δ_t
Δ_i
Δ_t
2
6
10
14
18
22
26
30
34
58
TIME
P
L
K
H

on the abscissa of the graph by an appropriate symbol. We see that AB and CO values nearly double upon the addition of lactoperoxidase. This is expected because lactoperoxidase is also a protein containing several tryptophan residues. Note that from this point on, AB remains essentially constant. In contrast, the addition of KI causes a rapid decrease in CO and PQ which rapidly reaches a plateau; Δ_t is characteristically twice as great as Δ_1. All fluorometric changes reach a plateau within five minutes of the addition of peroxide. The final data points (58 min.) indicate that little change in photodetectable activity occurs following the Δ_t phase of the reaction. A table with the dilution corrected data from the four iodination reactions is provided in Appendix C.

b. Analysis of fluorometric data.--Table 1A and Figure 2A present the fluorometric responses of the iodination reactions expressed as percent change of initial value. The initial value is defined as the CO or PQ value of the PRL plus lactoperoxidase solution immediately before the addition of KI; $\%\Delta_1$ is defined as the percent decrease in CO or PQ with respect to the initial value, six minutes after the addition of KI; $\%\Delta_t$ is defined as the decrease in CO or PQ eight minutes after the addition of H_2O_2. The data for the control experiments are also provided for comparisons (Table 1A and Figure 2B). The mean values ± standard deviations and the difference of the means were calculated. Note that in the complete iodination reactions, the changes in CO and PQ are proportionately the same, whereas in the control experiments CO decreases and PQ

TABLE 1.--Changes in fluororescence and partial quantum efficiency associated with iodination and iodide quenching.

A. Iodination reactions

	$\%\Delta_1$[a]		$\%\Delta_t$[b]	
	CO	PQ	CO	PQ
Lactoperox	-9.3	-9.5	-38.9	-40.4
	-8.8	-9.2	-33.3	-33.6
			-30.8	-30.9
			-34.7	-35.3
	-9.1 ± 1.6[c]	-9.3 1.4	-34.7 ± 3.7	-35.1 ± 3.8
Control[d]	-4.0	NC	-7.5	+4.2
	-7.3	NC	-9.0	+3.1
	-5.6 ± 2.6	NC	-8.3 ± 1.1	+3.6 ± 0.8

B. Iodide quenching

$\%\Delta_t$ CO[e]		
Molar Concentration of KI		
0.04 M	0.12 M	0.20 M
-24.7	-35.5	-46.9
-27.0	-35.4	-41.8
-25.8 ± 2.8[c]	35.5 ± 0.1	44.6 ± 5.6

[a] $\%\Delta_1$ = change in the CO or PQ following KI addition.

[b] $\%\Delta_t$ = total change in CO or PQ; measured 8 minutes after the addition of H_2O_2.

[c] Datum at bottom of each column = mean ± S.D.

[d] Control = lactoperoxidase-deficient reaction.

[e] $\%\Delta_t$ CO = change in CO as compared to iodide-free solution.

Figure 2.--Histogram of the percent change in fluorescence and partial quantum efficiency during iodination and iodide quenching.

A. Changes in CO and PQ during iodination.
Solid bar designates percent decrease occurring six minutes after the addition of KI ($\%\Delta_1$). Hollow bar designates the total per cent decrease as measured 8 minutes after peroxide addition ($\%\Delta_t$).

B. Changes in CO and PQ during lactoperoxidase-free control experiment.
Hollow and solid bars as in A.

C. Changes in CO during iodide quenching.
Numbers above each bar designate the molar concentration of KI.

n = sample size, vertical bars = S.D.

* = significantly different from control ($p < 0.05$).

** = significantly different from control and from Δ_1 ($p < 0.01$).

X = significantly different from initial value ($p < 0.05$).


A
n=4
CO
PQ
B
n=2
CO
PQ
C
n=2
0.04
0.12
0.20
x
x
*
**
**
20
10
0
10
20
30
40
% CHANGE, T½

increases. We see that in the control experiments Δ_t CO is considerably less than the experimental Δ_t CO.

B. Iodide Quenching

1. Objectives

Iodide alone is an effective quencher of tryptophanyl fluorescence (Lehrer, 1971). The objective of this experiment is to evaluate the collisional quenching response of PRL solutions to various concentrations of iodide. Data derived from such an experiment can be distinguished from the fluorescence quenching which accompanies iodination reactions.

2. Procedures

Solutions containing 25 µg/ml of o-PRL in 0.4 M sodium acetate plus either 0.00, 0.04, 0.12, or 0.20 M KI were prepared. Duplicate aliquots of each solution were then analyzed with an Aminco-Bowman spectrofluorometer with the excitation wavelength set at 288 nm and the emission wavelength set at 380 nm.

3. Results

Table 1B and Figure 2C present the fluorescence response of the various prolactin-iodide solutions expressed as a percent decrease of initial fluorescence. In this case, the initial fluorescence is defined as the fluorescence of the iodide-free prolactin solution. The mean ± standard deviations were calculated. The collisional quenching of PRL tryptophanyl fluorescence reported in Figure 2C appears to be directly proportional to the concentration

of KI. In order to obtain the same degree of quenching as occurred in the enzymic iodination reaction, nearly one million times the quantity of free iodide was required.

C. Discussion

In the linear amino acid sequence of the prolactin molecule as derived by Li (1970) tyrosine and tryptophan residues are well within 20 Å of each other, and thus capable of energy transfer. Moreover, the absorbance and fluorescence wavelengths of tyrosine and tryptophan are such that a substantial degree of energy transfer is anticipated. However, by selecting an excitation wavelength of 288 nm and an emission wavelength of 380 nm, tryptophan absorbance is favored and tyrosine fluorescence is excluded. Under these conditions, little energy transfer between the residues is possible. Thus, in the experiments reported here, the majority of the fluorescence data is believed to be derived from changes in the vicinity of the tryptophan residues only.

Figure 1 shows that the mechanism of the iodination reaction can be divided into two phases: pre- and post-peroxide injection. Although this division is somewhat artificial and not necessarily indicative of the normal sequence of events, it nonetheless defines two temporal and kinetically distinct phases of the reaction. The post-peroxide kinetics appear to be rapid and irreversible; being complete within three minutes. On the other hand, the pre-peroxide reaction appears to be at least partially reversible. A two-step reaction such as this would be consistent with the mechanism proposed by Morrison and Bayse (1970).

The compiled calculations of the iodination iodide quenching experiments (Figure 2) supply us with a great deal of information about the iodination reaction. The concentrations of KI used in the collisional quenching experiments are 5-6 orders of magnitude higher than those used in the iodination reactions; yet comparable tryptophanyl quenching occurs in both instances. It is therefore obvious that very different mechanisms are operative in the two reactions. Furthermore, the Δ_t CO (and PQ) changes during iodination are absolutely dependent on the presence of lactoperoxidase.

The fluorescence decrease which occurs in the absence of lactoperoxidase (Δ_1 in Figure 2B) is not clearly understood. It is unlikely that such low concentrations of KI could cause collisional quenching. However, Δ_1 CO which occurs during the control experiments is clearly distinguishable from Δ_1 CO in the iodination reaction. In the former case, there is no accompanying decrease in PQ, while in the latter case, the PQ decreases by more than 9%. Note that the addition of H_2O_2 to a solution of PRL and KI (no LPO) does cause a slight increase in PQ. Possibly, peroxide is capable of oxidizing indole bonds which causes a reduced absorbance at 288 nm and an increased PQ. This increase is significant ($p < 0.05$), and peroxide oxidation of protein bonds is probably of real consequence during routine iodination reactions. Another explanation of the increased PQ could be molecular stabilization due to the formation of disulfide bonds.

The significant decrease in PQ which occurs after the addition of KI to the iodination mixture is difficult to interpret, but

we suggest that a tertiary complex of PRL, lactoperoxidase, and iodide is formed. In addition, it is feasible that a reversible peroxide-independent iodination may occur during the Δ_1 phase of the reaction, but we have not yet confirmed this hypothesis by other techniques. Early investigations in our lab using Lugol's solution (unpublished results) produced similar, more exaggerated results. Thus, we suspect that excess lactoperoxidase may catalyze the reversible iodination reaction as well. We should note that the Δ CO and Δ PQ values during an iodination reaction are in very good agreement. This is simply another way of showing that absorbance remains essentially unchanged while tryptophan fluorescence is quenched.

The Δ_t phase of the reaction is strongly correlated with catalysis, but the interpretation is ambiguous. We considered four possible explanations. (1) Iodination may disrupt the transfer of energy from tyrosine to tryptophan either as a result of protein conformational change or as a result of iodide fixation on tyrosine. (2) Lactoperoxidase may catalyze the oxidation of tryptophanyl bonds. (3) Iodides bonded near or on tryptophan would cause an increase in radiationless deactiviation processes. (4) Changes in the protein conformation occurring during iodination may also change the micro-environment of the tryptophan residues. The first explanation is very unlikely because excitation and emission wavelengths were selected to exclude photometric contributions from tyrosine. The second alternative was first proposed by Alexander (1974). However, his procedure required an excess of peroxide equalling 10-100 times

the concentrations used in the present study. Furthermore, Alexander reported a concurrent decrease in absorbance at 280 nm which was not evident in this study. The third and fourth explanations both imply either stearic or conformational changes in the vicinity of the fluorophore. If the structure of the prolactin molecule is indeed altered during iodination, then we must question the biological integrity of the iodinated hormone. Furthermore, if tryptophan-90 is at or near the biologically active site, as was suggested by Kawauchi et al. (1973), then structural changes in this region could also alter biological activity. At present, we cannot distinguish tryptophan-90 from tryptophan-149, and the fluorescence studies reported here are not conclusive in confirming that changes occur at only one of these residues. However, radioiodinated prolactin is known to have a reduced biological potency (Frntz and Turkington, 1972; Posner, 1974; Rogal and Chrombach, 1975) which has not yet been adequately explained. Another consequence of conformational or stearic interference on the PRL molecule would be altered binding kinetics. Thus, kinetic constants derived from radioreceptor assays may not be indicative of the kinetics of the native hormone and its receptor.

II. Photometric Characterization of C$_8$11RAP Cells

A. Objectives

In these experiments we wanted to obtain excitation and emission spectra from suspensions of intact C$_8$11RAP cells, which could be compared to membrane fragment spectra published by other

investigators (Sonenberg et al., 1971; Wallach et al., 1970; Imae et al., 1975). We also wanted to establish a protocol of accurate data collection which could be used in subsequent experiments involving the addition of hormones. Ultimately, we hoped to correlate either AB, CO, or PQ to cell concentration so that the relatively inaccurate and time consuming method of counting cells with a hemocytometer could be avoided.

B. Procedures

All cell suspensions were checked for viability and counted in $Pucks_f$ plus glucose solution. Initial investigations produced relatively weak fluorescence signals for concentrations of 3×10^6 cells/ml at room temperature. Subsequently, the fluorescence chamber was cooled to 4°C with a continuous flow water bath. This step was taken to reduce the collisional quenching effects of solvent molecules and to reduce the rotational activity of the fluorophore itself. The result was about a 30% increase in CO and PQ values. Various concentrations of cells ranging from 0.5 to 6.0×10^6 cells/ml were prepared on three different days. Two (2.00) milliliter aliquots of the cells were pipetted into the sample cuvette and allowed to reach thermal equilibrium for 10 minutes. Excitation, emission and time scans were taken at various intervals. To assure even distribution of the cells, the cuvette was periodically agitated by inverting.

C. Results

Figures 3A and 3B are the excitation and emission spectra of $C_8$11RAP cell suspensions at 4°C. In the excitation scan the absorbance, fluorescence and PQ values are all plotted at different scale factors. Note in Figure 3A that the absorbance spectrum is broad and indistinct suggesting that light scattering may be responsible for a significant portion of the apparent absorbance. The maximal absorbance near 280 nm is typical of protein solutions. The maximal PQ occurs at 288 nm where the fluorescence efficiency is greatest. In Figure 3B excitation was at 275 nm and the broad characteristic spectrum which peaks at 335 nm is typical of tryptophan fluorescence. There is no shoulder at 303 nm suggesting that either tyrosine residues are absent, or more likely that energy transfer occurs between tyrosine and tryptophan. An essentially identical emission spectrum is produced when excitation is at 288 nm.

Time scans were taken every minute following the inversion of the cuvette. The photometric data were extremely variable between 0 and 3 minutes after the agitation of the cuvette. However, between 3 and 12 minutes experimental variation was less than ± 0.03 AB units, ± 2.5 CO units and ± 4.0 PQ units. After 12 minutes the cell suspensions were less stable and photometric values tended to decrease rapidly. If the cuvette was agitated at regular intervals, the initial photometric values could be re-established. Table 2 presents the AB, CO and PQ values obtained from cell suspensions prepared on three different days ± the experimental variation of three time scans taken at 4, 6 and 8 minutes following agitation. If the

Figure 3.--Excitation and emission scans of $C_8$11RAP cells.

A. Excitation scan from 250 to 320 nm; emission monitored at 335 nm.
AB = absorbance, CO = fluorescence, PQ = partial quantum efficiency.
All scales plotted with different scale factors: AB - 1000, CO - 10, PQ - 1.

B. Emission scan from 280 to 450 nm; excitation at 275 nm.

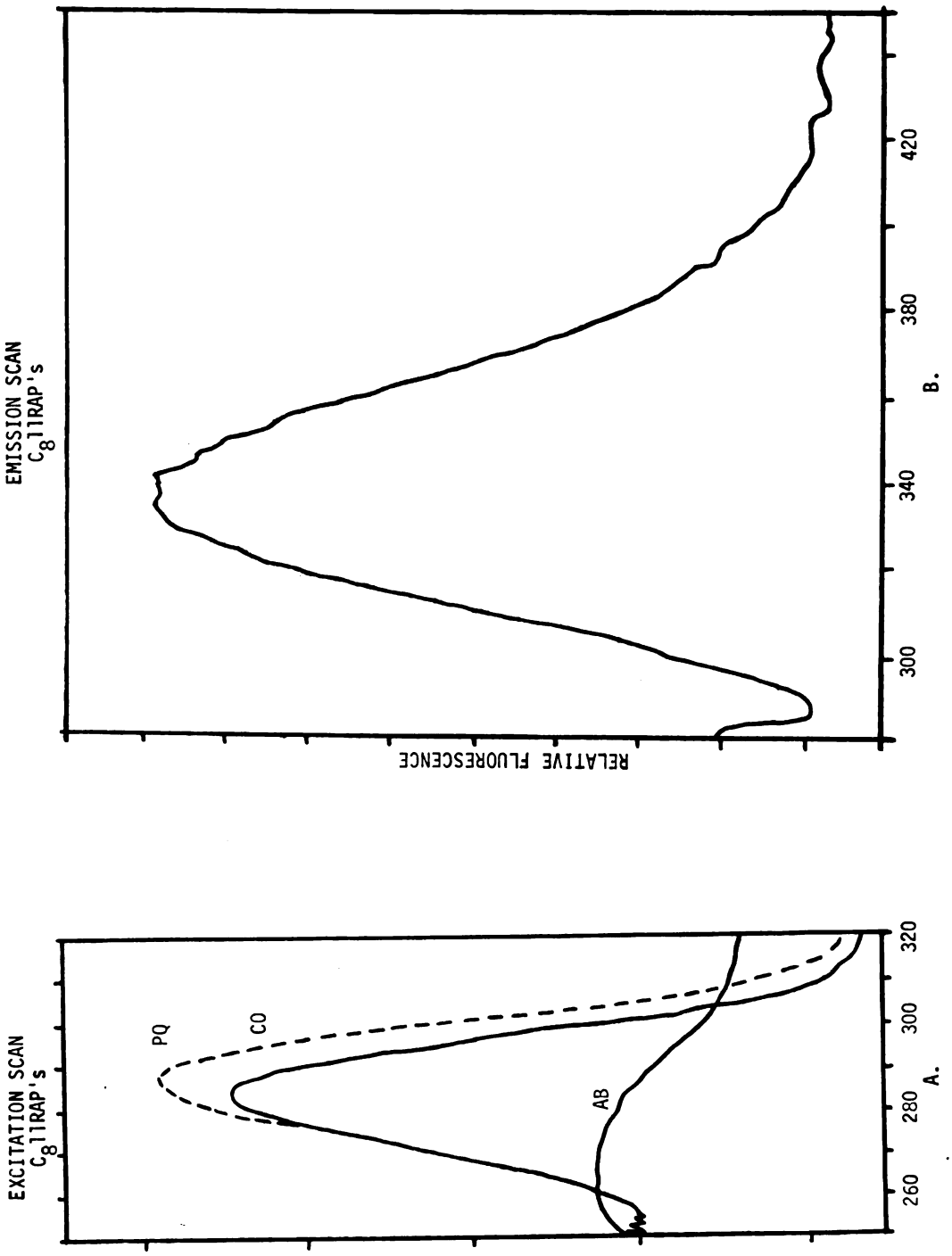

EMISSION SCAN
C_8 11RAP's
RELATIVE FLUORESCENCE
300
340
380
420
B.
EXCITATION SCAN
C_8 11RAP's
PQ
CO
AB
260
280
300
320
A.

TABLE 2.--Cell concentration and photometric data.

	AB[a]	CO	PQ	Concentration x 10^6 cells/ml
2[b]	.336 ± 0.01	100 ± 1	130 ± 2	0.45
1	.427 ± 0.9	141 ± 1	142 ± 2	0.86
3	.642 ± 0.02	204 ± 1.5	138 ± 2	1.82
1	.609 ± 0.02	225 ± 1.5	160 ± 3	3.41
2	.873 ± 0.02	314 ± 2.0	156 ± 4	4.27
1	.903 ± 0.03	424 ± 2.5	204 ± 5	5.25
3	1.280 ± 0.03	510 ± 2.5	174 ± 5	6.10

[a]AB, PQ, CO in arbitrary units ± experimental variance of 3 scans.

[b]Numbers 1, 2, 3 indicate the three different days of cell suspension preparation.

cell suspensions acted as pure fluorophores, the PQ values for all cell concentrations would be identical. The large deviation from a constant value is probably due to extraneous chromophores which were not washed free from the cell preparations, or peculiar concentration-dependent light scattering properties of the cells. It is apparent that CO is the only parameter which increases at regular intervals with respect to cell concentration.

Figure 4 is a plot of the CO values from Table 2 versus cell concentration. The correlation coefficient of 0.978 suggests a strong linear correlation, although the regression coefficient indicates a large degree of variability. The correlation developed from

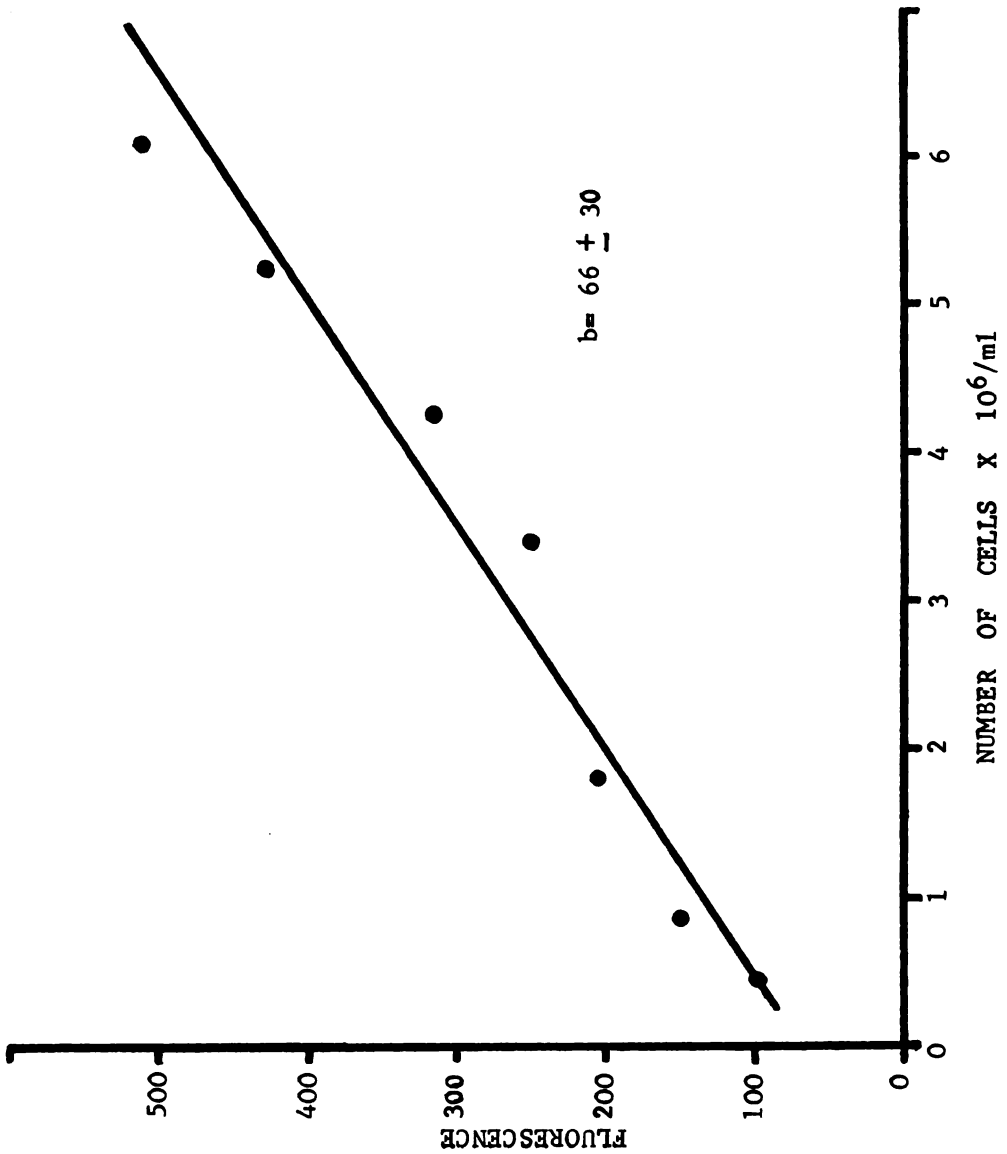
Figure 4.--Plot of $C_8$11RAP cell fluorescence as a function of cell concentration.

Fluorescence expressed in arbitrary units (10^{-5} M quinine sulfate in 0.1 M H_2SO_4 = 266.0 units). Viable cell concentation determined by dye exclusion and a Neubauer hemocytometer.

r = correlation coefficient = 0.978 ± 0.093.

b = regression coefficient = 66.0 ± 30.2
different from zero ($p < 0.1$)

FIGURE 4


FLUORESCENCE
500
400
300
200
100
0
0
1
2
3
4
5
6
NUMBER OF CELLS X 10^6/ml
b= 66 ± 30

these data was only intended to be used as a rough estimation of cell concentration and therefore a sample size of seven was considered adequate.

D. Discussion

An aqueous solution of tryptophan will produce a broad characteristic emission spectrum which peaks at 350 nm. When the polarity of the solution is reduced by adding organic solvents, the intensity of the emission is enhanced and the peak is shifted to a lower wavelength (blue shift, Bablouzian et al., 1970). Thus, we interpret the 335 nm peak of the $C_8$11RAP cells as an indication that a substantial portion of the membrane protein is located in a hydrophobic environment; probably embedded in lipid. Because no tyrosine fluorescence was evident, we assume that the efficiency of energy transfer to tryptophan was high. In order to avoid complex resolution of the data, we used an excitation wavelength of 288 nm which favors tryptophan absorbance for the time scan experiments. The emission wavelength of 348 nm in the time scans was purposely chosen to permit the study of a broader range of cell concentrations. That is, by choosing a wavelength somewhat off the peak, higher cell concentrations could be studied without exceeding the limits of the instrument.

Because the cell suspensions were stable (yielding relatively consistent data) between 3 and 12 minutes after agitation, the following protocol was developed for time scan studies. (1) Two (2.00) ml aliquots of cells were pipetted into the cuvette and allowed to

equilibrate at 4°C for ten minutes. (2) The cuvette was inverted and time scans (288//348) were taken at 4, 6 and 8 minutes following agitation. The AB, CO and PQ values obtained from these scans were averaged and the means ± experimental variance were recorded. (3) If data was needed over a longer period of time, step 2 was repeated. This protocol appeared to be adequate for the hormone treatment experiments which follow.

The low temperature used in these experiments (4°C) is comparable to that used by Imae et al. (1975) and has the advantage of reducing the metabolic activity of the cells. The lower temperature also enhances the quantum efficiency of the fluorophore by reducing collisional quenching and rotational activity. During cooling, CO values increase while AB values remain relatively constant and thermal transcience can be detected as changes in PQ. Thus, time scans were taken only when PQ values remained within ± units.

Ultimately, we wanted to detect changes in fluorescence which accompany hormone-cell interactions and it was necessary to develop a method of cell concentration analysis which was rapid and appropriate to our experimental protocol. Methods such as protein (Lowry et al., 1951) or DNA (Burton, 1956) determinations were not feasible because rapid and on line adjustments of cell concentration were essential. Thus, we hoped that cell concentration could be correlated to one of the photometric parameters which were routinely measured. This would allow us to dilute or concentrate the cell suspensions prior to any experimental manipulation. We see in Figure 4 that a strong linear correlation is obtained between CO and

cell concentration. The slope of the least squares line, however, is only different from zero at the 90% confidence level ($p < 0.1$). Much of the variation around the regression line is probably due to errors in cell counting with the hemocytometer. In our lab, we rarely obtain cell concentration estimates which are closer than ± 5% from 5 successive trials. A casual examination of Table 2 indicates that AB and PQ are poor indicators of cell concentration. This is probably an artifact of the method of cell preparation. From day to day, minute but differing quantities of growth medium remain in the washed cell suspensions. Although many of these contaminants may absorb at 288 nm, only a few of them will fluoresce at 348 nm. The result is a variable AB value and a relatively consistent CO value for a given concentration of cells. And because AB fluctuates, PQ also fluctuates making both of these data difficult to use experimentally. In subsequent studies, Figure 4 was used as a standard estimate of cell concentration. Although the estimates may be approximate, they provide a reasonable assessment of cell density which helps to define the parameters of the experiment.

III. Fluorescence Monitor of Hormone-Cell Interactions

A. Objectives

The tryptophanyl fluorescence quenching of rat liver membranes due to b-GH has been correlated to ATPase activity of the cell (Rubin et al., 1973; Postel-Vinay et al., 1974). Similarly, TRH binding to anterior pituitary cell membranes has been correlated to fluorescence changes of membrane proteins (Imae et al., 1975). In

this study, we wished to evaluate changes in membrane fluorescence which accompany the incubation of intact pituitary cells with various concentrations of o-PRL and o-GH.

B. Procedures

Solutions of o-PRL, o-GH and $C_8$11RAP cells were prepared in $Pucks_f$ plus glucose according to the methods described above. For each experiment, duplicate 2.0 ml aliquots of cells were scanned according to the protocol described in the previous section. To one aliquot, 250 µl of $Pucks_f$ plus glucose was added, and to the other aliquot an equal volume of either o-PRL or o-GH was added. A number of scans were taken following the addition of hormone. Scans were also made of hormone solutions in the absence of cells. All experiments were conducted at 4°C. The units of fluorescence in all studies were standardized against 10^{-5} M quinine sulfate in 0.1 M H_2SO_4.

C. Results

Table 3 lists the fluorescence of various concentrations of o-GH and o-PRL in cell-free solutions of $Pucks_f$. The experimental variation was derived from three separate trials of 250 µl of hormone injected into 2.0 ml of $Pucks_f$ solution. Note that the fluorescence values are approximately linearly related to the concentration of hormone. The variation is more likely a function of solution preparation than of instrumental error.

When hormone was added to the initial cell suspensions time scans were taken every minute for fifteen minutes and then every five

TABLE 3.--Fluorescence of ovine prolactin and ovine growth hormone.

	o-PRL g/ml							o-GH g/ml	
	17.5	22.0	25.0	30.0	45.0	50.0	100.0	25.0	45.0
CO	16.1	21.0	23.7	28.0	42.0	47.2	91.4	14.4	26.0
±E.V.	±0.2	±0.2	±0.3	±0.2	±0.2	±0.2	±0.2	±0.2	±0.2

CO ± E.V. = Fluorescence in arbitrary units ± experimental variance.

to ten minutes for up to three hours. Periodically, emission scans were also taken before and after the addition of hormone. Detectable fluorescence changes were complete within ten minutes and remained constant for at least three hours. The fluorescence values computed for each experiment were taken from the 9, 11 and 13 minute time scans and expressed as the arithmetic mean ± experimental variance. Figure 5 is a plot of the fluorescence response of various cell concentrations to three different concentrations of o-PRL. The abscissal values were estimated from the standard plot of initial CO vs. cell concentration (Figure 4). Each point represents the results of a single experiment. The ordinate values were derived from the following equation:

$$Y = CO_{exp} - CO_{control} \quad (1)$$

where

CO_{exp} = the fluorescence of the hormone and cell solution minus the fluorescence due to hormone (from Table 3), and

$CO_{control}$ = fluorescence of the duplicate cell suspension plus 250 µl of $Pucks_f$.

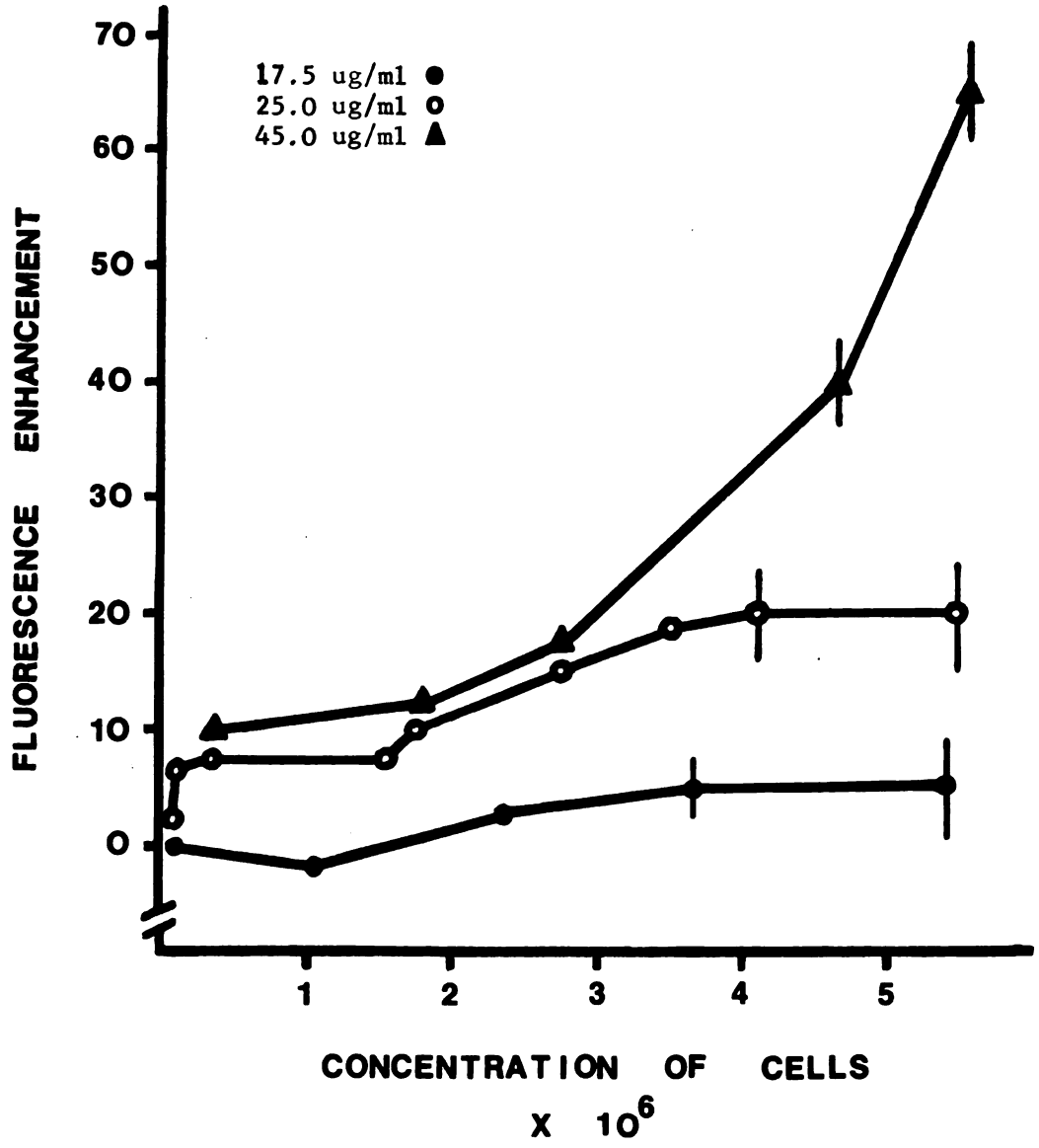
Figure 5.--Fluorescence enhancement as a function of initial cell concentration.

Each point is the result of a single experiment. Vertical bars indicate experimental variance of 3 time scans. Cell concentrations were estimated from standard curve of cell concentration vs. CO.

Fluorescence enhancement = $CO_{exp} - CO_{control}$.

Units are arbitrary.

o, ● and ▲ = final concentration of hormone.


17.5 ug/ml
25.0 ug/ml
45.0 ug/ml
FLUORESCENCE ENHANCEMENT
70
60
50
40
30
20
10
0
1
2
3
4
5
CONCENTRATION OF CELLS
X 10^6

The duplicate cell suspensions had equivalent initial fluorescence values within experimental error, generally ± 2.0 units. The experimental variance of the derived values (Y) were taken as the sum of the CO_{exp} and the $CO_{control}$ experimental variances (see Appendix D for tabular listing of data). The three graphs generated in Figure 5 demonstrate the dependence of fluorescence enhancement both on cell concentration and hormone concentration. At low cell densities greater hormone concentrations are needed to produce a response. The 17.5 µg/ml and 25.0 µg/ml curves can both be fit to regression lines ($r > 0.85$), but only the 25.0 µg/ml curve has a slope which is significantly different from 0 ($p < 0.1$). The curvilinear appearance of the 45 µg/ml plot led us to believe that a log-dose rather than a linear relationship existed between fluoresence enhancement and cell concentration. It is also apparent that the greatest changes occur at higher cell concentrations.

Figure 6 is a dose-response curve of fluorescence versus prolactin concentration for a given concentration of cells. The ordinate values were obtained from equation (1), and each point represents the results of a single experiment. It was extremely difficult to pipette the 20 identical aliquots for the dose-response experiments, and a wider experimental variance was tolerated. The initial CO values of the cell suspensions varied from 290 to 330 units yielding a mean concentration of $3.65 \pm 0.26 \times 10^6$ cells/ml (mean ± standard deviation; see Appendix E). The resulting graph has a distinct sigmoid shape with an exponential rise between the concentrations of 18 and 30 µg/ml o-PRL. The slope decreases

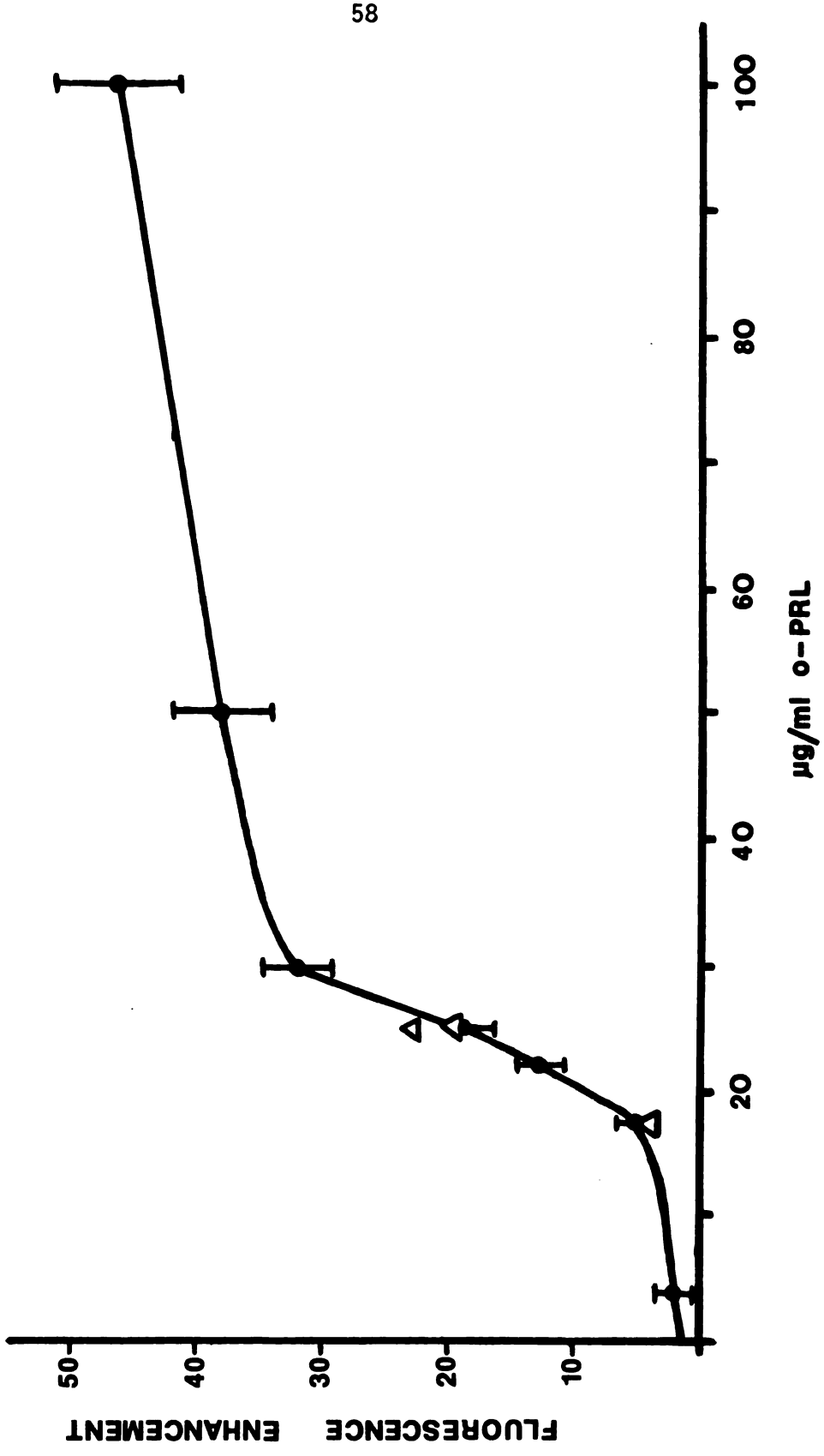
Figure 6.--Fluorescence enhancement as a function of prolactin concentration.

Each point is the result of a single experiment. Vertical bars indicate experimental variance of 3 time scans. Concentration of cells for each experiment = $3.65 \pm 0.26 \times 10^6$ cells/ml.

Fluorescence enhancement = $CO_{exp} - CO_{control}$.

Units are arbitrary.

● and Δ = two different series of experiments.


FLUORESCENCE ENHANCEMENT
10
20
30
40
50
20
40
60
80
100
µg/ml o-PRL

rapidly above 50 µg/ml but continues to rise until 100 µg/ml. Similarly, there is a gentle decrease in slope between 17.5 and 3 µg/ml. Such a graph would be consistent with what one expects in a positively cooperative system.

Table 4 presents the pooled results of two or more experiments with various concentrations of hormone and 3.65×10^6 cells/ml. The mean and standard deviation for each hormone treatment were calculated and compared statistically. The data compiled for PRL treatments in Table 4 correspond to the exponential phase of Figure 6. In spite of the small sample number, all of the means are significantly different from each other at the 99% confidence level. The statistics employed in this analysis assume Gaussian distribution of the individual trials which is a questionable assumption for data derived from a dose response curve; especially in the case of the pooled 45-50 µg/ml datum. However, to a first approximation the Student's t test provides a reasonable description of the results. Note that no significant response is elicited by cells treated with 25 µg/ml o-GH while a 45 µg/ml treatment causes fluorescence quenching.

D. Discussion

The fluorescence of photoexcited cell membranes represents the emissions from a heterogenous population of surface proteins. Although these proteins cannot be distinguished solely on the basis of their colorimetric properties, they can be distinguished functionally. Thus, only a given proportion of the proteins will be

TABLE 4.--Fluorescence response of C$_8$11RAP cells to ovine growth hormone and ovine prolactin (288//348).[a,b]

1	2	3	4	5
25 µg/ml o-GH	45 µg/ml o-GH	17.5 µg/ml o-PRL	25 µg/ml o-PRL	45-50 µg/ml o-PRL
1.3	-8.5	5.0	18.6	31.0
0.5	-6.0	4.5	19.0	29.4
-1.0			22.2	
0.27±1.03 n = 3	-7.25±1.77[c] n = 2	4.75±0.35[c] n = 2	19.93±2.02[c,d] n = 3	30.2±1.1[c,d,e] n = 2

[a]All values = mean ± S.D. (CO_{exp} - CO_{cont}).

[b]Each datum represents the result of a single experiment with 3.65 ± 0.26 x 10^6 cells/ml, and was derived from equation (1).

[c]Different from 4 ($p < 0.01$).

[d]Different from 3 ($p < 0.01$).

[e]Different from 4 ($p < 0.01$).

involved in any given physiological activity. If the biological activity necessitates a conformational or structural change in the protein, then a concomitant change in fluorescence may also occur (i.e., quenching, enhancement, peak shigt, etc.). Polypeptide hormones are known to have membrane bound protein receptors in a variety of target tissues (Frantz et al., 1974; Shiu and Freisen, 1975; Cuatrecasas, 1974) which doubtlessly contribute to the total fluorescence emitted from photoexcited membranes. The interaction of prolactin with its receptor is believed to be analagous to enzyme-substrate binding (Shiu and Freisen, 1974) and thus probably involves tertiary changes in either the hormone or the receptor or both.

Consequently, we would anticipate fluoremetric changes in membrane emissions during PRL binding. The changes, however, are not directly proportional to the total membrane fluorescence but to the proportion of the total fluorescence which is contributed by the binding site alone. It is therefore misleading to express the fluorometric changes as a percent of the total.

In the present study, an additional complicating factor is involved. The concentrations of hormone used also possess a significant intrinsic tryptophanyl fluorescence (Table 3). We are interested in the response of the membranes alone, yet are unable to distinguish hormone from membrane fluorescence. The recorded fluorescence enhancements (Figures 5 and 6) in some cases equals more than twice the fluorescence of the hormone alone. If the enhancement arose from changes in the hormone, then PRL would be required to double its quantum efficiency. On the other hand, if the enhancements reflect changes at the membrane, a lesser, more plausible augmentation of membrane quantum efficiency would explain the fluorescence enhancement. Both explanations are reasonable; unfortunately, we cannot yet resolve which (if not both) mechanisms are operative.

Throughout these experiments, periodic emission scans were taken both before and after the addition of hormone. Although the intensity of fluorescence varied according to the experimental procedure, there was no evidence of an emission peak shift. Since the peak emission was not shifted, the environment surrounding the fluorescent tryptophan residues probably is not greatly changed in polarity. Thus, fluorescence enhancement would be caused only by

(a) a conformational change in the membrane whereby a tryptophan group is brought near to a tyrosine (energy transfer), (b) non-radiative competitive quenching processes were altered, or (c) the hormone binding reaction causes an increased conformational organization of the hormone or the receptor.

We see in Figure 5 that high concentrations of cells (about 3.6×10^6/ml) are necessary before dose dependent changes in fluorescence are resolvable. This may be due to the existence of relatively few PRL specific binding sites per cell. There are, however, certain trends which seem to be present which merit some discussion. For PRL doses of 25 and 17.5 µg/ml a plateau region on the graph is distinguishable between cell concentrations of 3.5 and 5.5×10^6 cells/ml. Note that the graph of 25 µg/ml of PRL appears to be shifted upward and to the right with respect to the 17.5 µg/ml graph. In contrast, the graph of 45 µg/ml of PRL undergoes a rapid rise over the same concentration ranges. The significance of these phenomena was not clear, so we decided to try dose-response experiments which are illustrated in Figure 6. At a constant concentration of 3.65×10^6 cells/ml the fluorescence enhancement undergoes the most dramatic changes between 18 and 30 µg/ml o-PRL. At higher concentrations (30-100 µg/ml), a less pronounced augmentation occurs which may continue indefinitely. When experiments with comparable cell and hormone concentrations were pooled (Table 4), we found that the mean fluorescence enhancement values in the 17.5, 25 and 45 µg/ml treatments were all significantly different from each other. Thus, the protocol and instruments employed in these experiments were sufficient

to resolve fluoremetric changes resulting from small changes in hormone concentration. We also see in Table 4 that the response of $C_8$11RAP cells to o-GH is opposite to that of o-PRL. That is, o-GH causes a quenching of fluorescence at 45 µg/ml doses while o-PRL causes enhancement. If both of these responses are due to conformational changes in specific membrane receptors, then two distinct populations of binding sites can be identified by these techniques. Quenching is associated with o-GH binding and enhancement is correlated with o-PRL binding. At this time, no o-GH binding sites have been identified on $C_8$11RAP cells by radioreceptor methods, but tryptophanyl quenching does occur in rat liver membranes exposed to b-GH (Postel-Vinay et al., 1974).

We are very pleased with the ability of the spectrofluorometer to resolve changes in membrane conformation associated with small changes in hormone concentration. However, it is unknown whether this system will be applicable to other target tissues and lower concentrations of hormone. Optimally, we would like to detect changes in membrane components in response to picomolar concentrations of PRL.

Although Frantz et al. (1975) have demonstrated specific binding to $C_8$11RAP as well as normal pituitary cells, no physiological response has been identified. It was postulated that PRL binding may be the stimulus for a short loop feedback mechanism. In this study, fluorescence enhancement has been correlated to o-PRL cell interactions, which suggest that the hormone is capable of stimulating conformational changes in the membrane or hormone

protein structure. Concentrations of 18-30 µg/ml of o-PRL appear to be a critical range through which the largest changes in fluorescence occur. Quite possibly, the changes occurring at the membrane at these specific hormone concentrations could trigger intracellular mechanisms which function to regulate such cytoplasmic processes as transcription or secretion. Although microgram concentrations would be considered extremely high as compared with normal physiological levels (2-200 ng/ml), microgram concentrations might be expected in the capillaries bathing the anterior pituitary. Furthermore, since $C_8$11RAP cells secrete prolactin (Sonnenschein et al., 1974), they are constantly exposed to relatively high levels of hormone. It is not surprising that even higher levels are necessary to initiate the binding process. This hypothesis was indirectly supported by experiments conducted in our lab with o-PRL and partially purified membrane particles (unpublished data). We found that comparable fluorescence changes could be effected by reacting the liver membranes with 100 ng/ml of hormone. We are presently planning to obtain cultures of liver and mammary cell lines to be studied in the future.

The fluorescence response of the PRL-cell association appears to be very rapid; being complete within 10 minutes. If fluorescence is a monitor of receptor binding, then the kinetics are very rapid. This evidence lends support to the work of Frantz et al. (1974) and appears to contradict the studies by Shiu and Friesen (1974). Alternatively, the fluorescence response may only monitor the initial phases of PRL-receptor association, giving a misleading estimate of the reaction time. However, because the PRL used in this study

was not altered by iodination, we feel that the system provides a more accurate reflection of physiological conditions. In addition, the cells are intact and metabolically active, whereas the bulk of the radioreceptor work was done with cell homogenates or purified membrane preparations whose structure may no longer be like that of the intact cell.

The 4°C temperature used throughout these experiments may have had a significant effect on the results. Sonenberg (1969) found that erythrocyte membranes were only responsive to h-GH in the range of 21-40°C, being maximal at 37°C. Similarly, Shiu and Friesen (1974) found that radioreceptor binding to rabbit mammary homogenates was greatly decreased by low temperatures. It would be very informative to evaluate the effects of temperature on fluorescence-monitored binding reactions.

The tendency of intact cells to settle out of solution was a serious problem throughout the study. In order to obtain consistent results, the fluorescence cuvette had to be agitated every 8-12 minutes. An important step in the refinement of this system would be the development of a matrix which would immobilize the cells, be light transparent and permeable to hormone solutions. Possibly a polymeric gel such as Ficol™ should be investigated.

IV. Light Scattering of C8llRAP Cells

A. Objectives

The surfaces of individual or aggregates of cells are large relative to the wavelengths of the incident radiation used in the

fluorescence experiments and therefore will reflect a substantial portion of the excitation radiation. If ignored, these surface reflections (Tyndall light scattering) may produce inappropriate absorbance and fluorescence values which can lead to incorrect interpretation of photometric phenomena (Willard et al., 1970). In addition, the proteins on the surface act as conglomerates of oscillating charges which can disperse some of the incident photoenergy in directions other than the direction of the incident radiation (Rayleight light scattering; Van Holde, 1971). Both of these effects were presumed to be operative during the experiments described in the previous section. Since Tyndall light scattering is purely a reflective process, the intensity of the scattered light is directly proportional to the surface area and density of the scattering centers. Unfortunately, cell suspensions are a heterogenous mixture of individual and aggregates of cells and we suspected that the relative abundance of disaggregated cells increases as the suspensions were diluted. In the fluorescence experiments, 0.25 ml injections of hormone and control solutions were made directly into the fluorescence cuvette which meant that the cell suspensions were diluted by a factor of 0.888. This dilution may have disrupted cell aggregates and therefore decreased both the density and the surface area of the scattering particles. Furthermore, because we postulated that membrane proteins undergo conformational changes during the hormone binding process, the Rayleigh light scattering properties of the $C_8$11 cells may have also changed. Thus, we felt it was essential to

evaluate the light scattering properties of the cells under the conditions of the earlier fluorescence experiments.

B. Procedures

The light absorbance of $C_8$11RAP cells falls off rapidly at wavelengths above 325 nm and thus by monitoring light scattering of monochromatic light at 330 nm, an absorbance artifact can be avoided. In these experiments individual 2.0 ml aliquots of various concentrations of cells were pipetted into the sample cuvette of the spectrofluorometer and allowed to equilibrate for ten minutes at 4°C. The collection of data and protocol was identical to that used in the fluorescence experiments except that both monochrometers were set at 330 nm. The 90° emission data were collected both before and after the addition of hormone or $Pucks_f$ at the intervals specified above. In the light scattering experiments, duplicate suspensions were not compared, but a series of cell concentrations were injected with either o-PRL (final concentration of 40 μg/ml) or $Pucks_f$.

C. Results

The intensity of scattered light (330∫∫330) at the cell concentrations used in the fluorescence experiments tended to be higher than the intensity of fluoresced light (288∫∫348). Thus, after determining the cell concentration for the standard curve (Figure 4), the voltage applied to the emission photomultiplier amplifier was reduced from 8.0 to 6.5 Kvolts. This had the unfortunate effect of changing the arbitrary value of the light scattering units. However, the relative effects of the dilution phenomenon are still evident,

though the absolute value of the units is different from those used in the fluorescence experiments. A 40 μg/ml solution of o-PRL in $Pucks_f$ plus glucose also produces a small but detectable amount of light scattering. In three consecutive trials, the solution produced a light scattering of 2.6 ± 0.4 units (mean ± experimental variance). Thus, in order to evaluate only the changes in membrane light scattering, 2.6 units were subtracted from the final light scattering values for C_8ll cell suspensions.

Figure 7 is a plot of the decrease in light scattering which occurs following the addition to various initial concentrations of cells of 250 μl of PRL or $Pucks_f$ plus glucose. The ordinate values were derived by subtracting the diluted cell suspension light scattering value from the initial light scattering value. Thus, the graph <u>is not</u> analogous to Figure 5. The solid line denotes the decrease which is anticipated for the dilution of homogenous suspension of light scattering particles. Note that at low cell concentrations, there is very good agreement with the theoretical line, but at concentrations above 1.3×10^6 cells/ml, a dilution affect gives rise to a disproportionate decrease in light scattering. Within experimental variance (vertical bars) there was no detectable difference between the light scattering changes in the hormone (o) and the $Pucks_f$ (●) injected treatments. Thus, if the $Pucks_f$-injected light scattering values were subtracted from duplicate o-PRL-injected values, the result would be 0 for all concentrations of cells. It should be remembered that only the 90° emissions can be monitored with this instrument. It is assumed that unobserved changes in

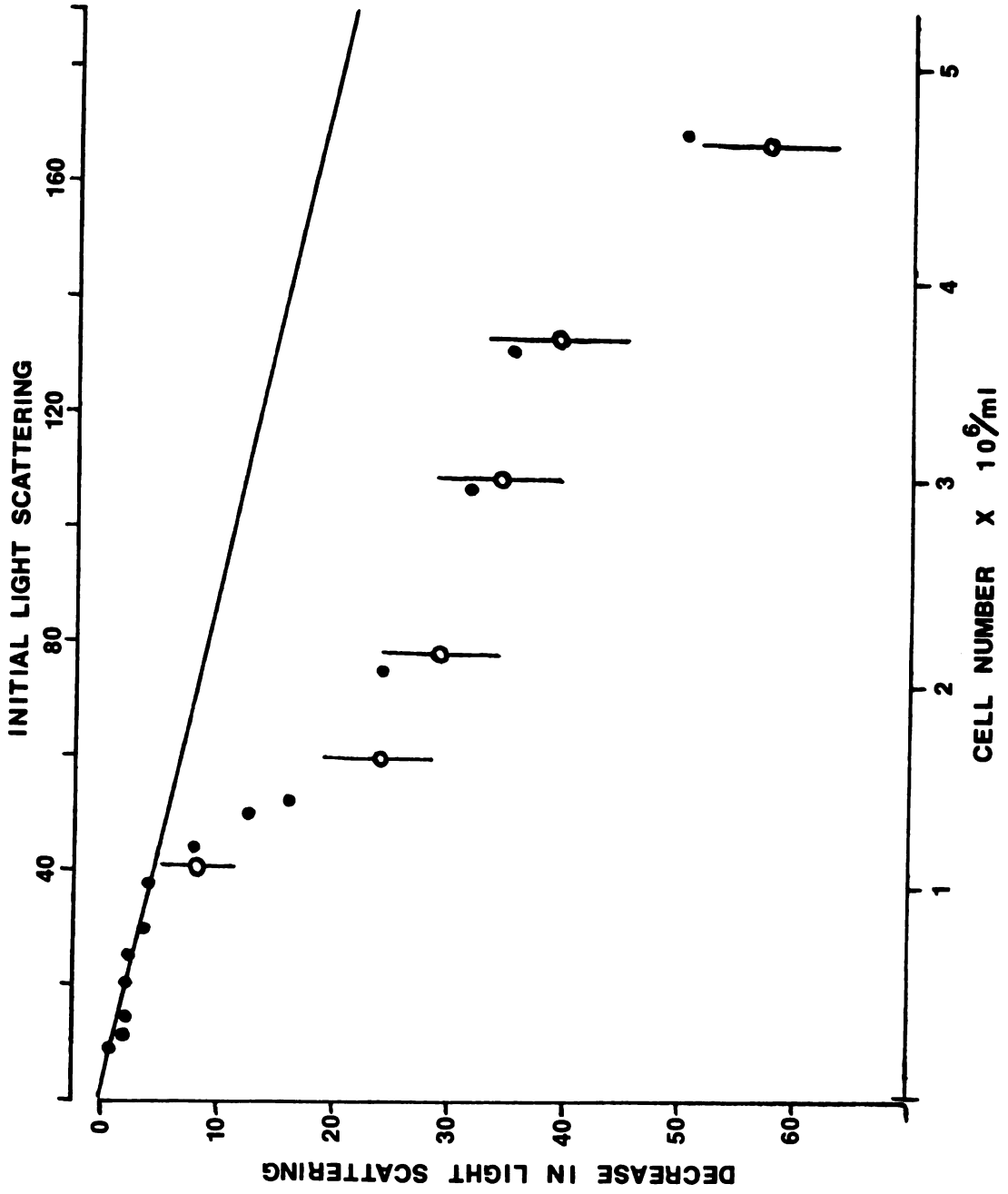
Figure 7.--Changes in light scattering of $C_8$11RAP cells due to dilution.

Decrease in light scattering = Initial - Final.

● = dilution with $Pucks_f$.

o = dilution with $Pucks_f$ + o-PRL: final concentration 40 µg/ml.

Light scattering units are arbitrary.

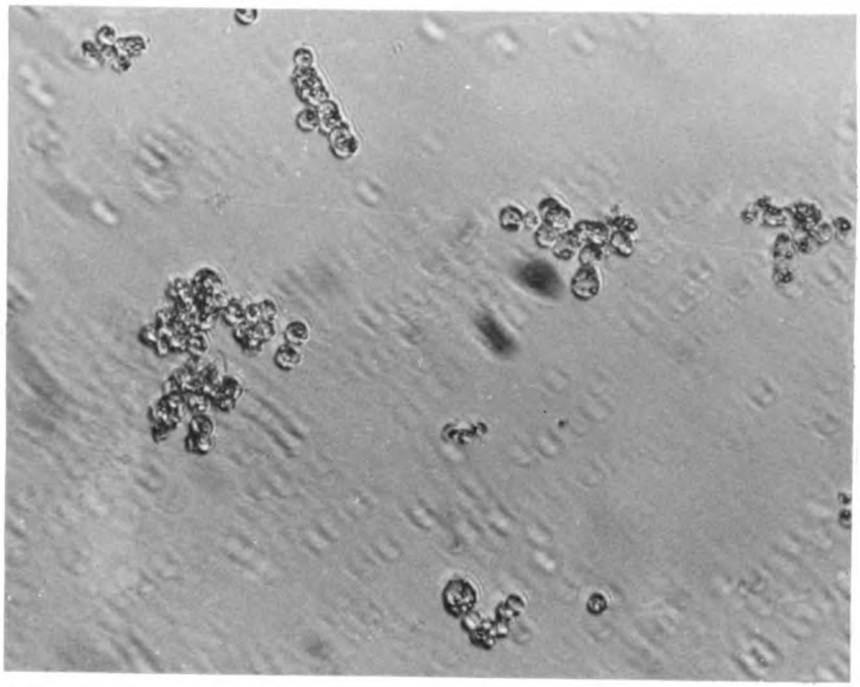

INITIAL LIGHT SCATTERING
40
80
120
160
DECREASE IN LIGHT SCATTERING
0
10
20
30
40
50
60
CELL NUMBER X 10⁶/ml
1
2
3
4
5

Rayleigh light scattering occur at other angles, but are proportionate and randomly distributed.

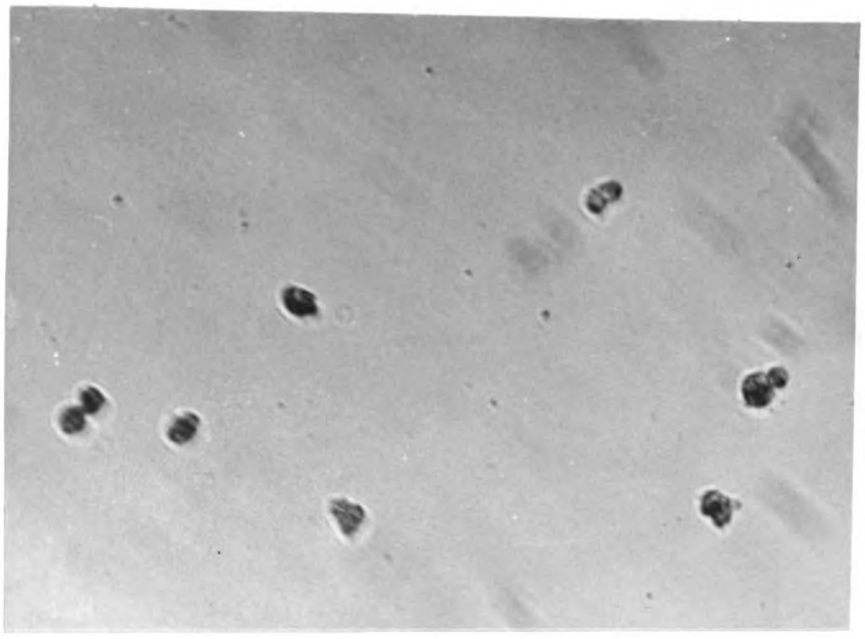
Photographs were taken of cells suspended in culture medium at two different concentrations. In Figure 8A the cell concentration was approximately 2.0×10^6 cells/ml. We see that most of the cells exist as aggregates of five or more cells and very few free cells are present. Figure 8B is a photograph of the same solution after diluting by a factor of four and agitating with a glass pipette. In this case, most of the cells are in groups of 2 or 3 and more individual cells are present. Presumably, the disaggregation was a direct result of dilution.

D. Discussion

The light scattering data obtained in these experiments provide us with valuable information concerning the treatment of intact cell suspensions in fluorescence experiments. We see that the dilution of cell suspensions at concentrations greater than 1.3×10^6 cells/ml will cause a disproportionate decrease in light scattering. We also found in the control fluorescence experiments that a disproportionate decrease in light emitted at 348 nm accompanied the addition of 250 µl of $Pucks_f$. Because the light scattering and the apparent fluorescence decreases were proportionately equivalent, we believe that both phemonema were due to changes in Tyndall light scattering alone. We furthermore demonstrated (Figure 8) that $C_8$11RAP cells exist both as aggregates and free cells and the relative abundance of each appears to be related to the cell concentration.



A. 2.0×10^6 cells/ml.



B. 0.5×10^6 cells/ml.

Figure 8.--Photomicrographs of $C_8$11RAP cells.

Therefore, we feel that it is reasonable to postulate that the light scattering phenomena is a result of the disaggregation of cells. That is, when a cell suspension is diluted, not only are the scattering centers dispersed but the reflective area of each center is also reduced.

Although the Tyndall perturbations are real and measurable, there is no distinguishable difference in light scattering with the PRL or control vehicle that is injected in the cell treatments. It should be noted, however, that the experimental variance was rather large in the light scattering experiments such that small differences may have been obscured. If any trend does exist, it would be that PRL seems to augment the decrease in light scattering as compared to vehicle-injected suspensions. Possibly this is the result of Rayleigh effects which accompany conformational changes of the surface proteins. This would be opposite to the fluorescence effect described in the previous section. But as a first approximation, it is sufficient to assume equivalent light scattering decreases. We must remember that only the 90° emission was monitored and, thus, larger and undetected light scattering changes may have occurred at smaller angles with respect to the incident light beam. These, however, would have no effect on the fluorescence measurements as reported.

In conclusion, we see that Tyndall light scattering changes constitute a serious experimental error in fluorescence studies of $C_8$11RAP cells. However, by expressing fluorescence enhancement as

the difference between duplicate samples of hormone and control injections, an internal correction is made (equation 1). In retrospect, it would be preferable to establish an experimental design which did not depend on the dilution of the cell suspensions.

SUMMARY AND GENERAL DISCUSSION

Lactogenic hormones and their receptors have been the subject of intense investigation during the past five years. We now know that prolactin binds specifically to a variety of tissues including mammary, liver, adrenals, prostate, seminal vesicles and the pigeon crop sac. The receptor itself is located on the membrane surface; is protease sensitive, neuraminidase indifferent and has been partially purified. Most of the work with the radioreceptor assay has relied on the lactoperoxidase catalyzed iodination reaction for ^{125}I fixation to the native hormone.

In this study, we have demonstrated specific quenching of tryptophanyl fluorescence of o-PRL which is associated with the lactoperoxidase catalyzed iodination. The fluorescence quenching (34.7%) is rapid, irreversible and clearly distinguishable from collisional quenching. We believe the fluorometric effect to arise from (1) iodide fixation on or near tryptophan, or (2) conformational changes in o-PRL tertiary structure. In addition to their fluorescent behavior, one of the two tryptophans (Try-90) is believed to be located on or near the biologically active region of the hormone (Kauwachi et al., 1973). Thus, stearic or conformational changes at this locus could have serious consequences for the biochemical potency of the molecule. Unfortunately, the radio receptor and radioimmunoassays (RRA and RIA) operate under the assumption

that the ^{125}I-labelled hormone is functionally identical to the native hormone. Similarly, the Scatchard analysis relies on equivalent competition of the labelled and unlabelled hormone for the same population of receptors. In light of the present findings, we are compelled to question these fundamental assumptions. It is therefore reasonable to question the physiological significance of the 10^{-10} to 10^{-9} M dissociation constants which have been estimated for the various PRL-receptor complexes.

Using fluorometric methods we have also monitored the interaction of o-PRL with intact rat anterior pituitary tumor cells which are known to possess specific and saturable binding sites (Frantz et al., 1975). The system which we developed is capable of detecting tryptophanyl fluorescence enhancement from membrane proteins of $C_8$11RAP cells in response to micromolar concentrations of o-prl. The effect is specific to o-PRL as compared to o-GH. The exponential rise of a sigmoid dose-response curve occurs between 18 and 30 µg/ml of hormone. It appears that a threshold is reached at a concentration of approximately 18 µg/ml which potentiates the binding of additional molecules of hormone. It is difficult to compare these results to those obtained from RRA methods, because the experimental designs differ. In the RRA, specific binding is defined by the capacity of native hormone to displace or compete with labelled PRL. In contrast, the fluorescence assay uses only native hormone and the specificity of the response is defined by comparison to the zero control and the effects of o-GH. Although the former method permits easier quantitation under ideal conditions, the latter method does not

depend on a chemically altered hormone. In spite of these differences, there are several observations which should be discussed. With the fluorescence method we have estimated a saturation time of approximately 10 minutes. This datum was derived from a system utilizing intact, metabolically active cells and native hormone. Furthermore, the photophysical response is the direct result of a macromolecular conformational change. The reaction time is also consistent with those obtained by Sonenberg (1969), Postel-Vinay et al. (1974) and Imae et al. (1975). In contrast, the RRA generally depends on experimental methods using cell homogenates, indirect calculations of specific binding and a particular batch of labelled hormone. By using disrupted cells, one risks exposing the hormone and receptor to intracellular enzymes which may destroy a significant portion of the tissue's receptor activity. Moreover, the response is usually defined as the difference of total and nonspecific binding and the 90% saturation times are equivocal (Frantz et al., 1974; Shiu and Friesen, 1974; Nicoll, 1975). We have also found in our lab that the fluorescence method produces more consistent results than the RRA (personal observation). Although we have not exploited its potential, it appears that the fluorescence method may be capable of distinguishing at least two populations of binding sites simultaneously. That is, the sequential addition of o-PRL and o-GH to a suspension of $C_8$11RAP cells should elicit fluorescence enhancement and fluorescence quenching, respectively.

It is perhaps unfortunate that anterior pituitary cells were used for these initial studies because masking of binding sites by

endogenous r-PRL may have given misleading results. At present the sensitivity and general applicability of the fluorescence method is unknown. However, the success of the experiments of the Sonenberg and Tashjian groups with b-GH, TRH and various membrane fractions is a promising incentive for the development of intact cell systems. The ultimate success of the fluorescence assay will depend on more extensive investigations with liver and mammary cell lines. Methodological refinements should also be explored because light scattering changes associated with cell suspension dilution are a possible source of error.

Apart from the endocrinological aspects of this study, we have obtained supporting evidence for the theory of a dynamic membrane. Although there is some ambiguity in the interpretation of the data, it is likely that PRL and GH both confer their biological activity by causing conformational changes in specific membrane proteins. At this time we have not ascertained what cytoplasmic processes are linked to the membrane phenomena, but a feedback mechanism is certainly operational and should be further studied.

APPENDICES

APPENDIX A

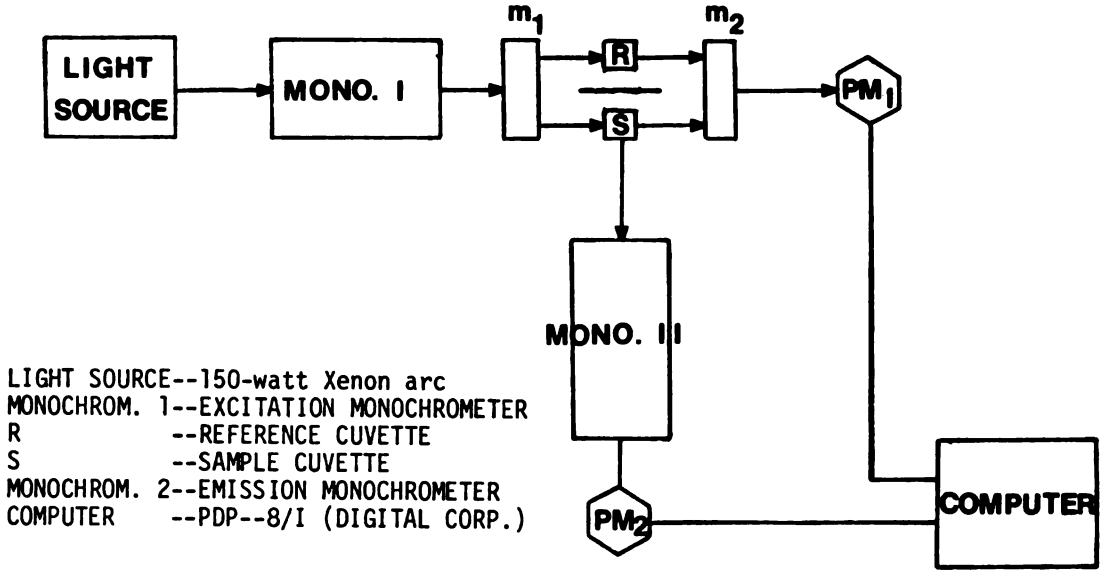


Figure 9.--Optics and technical specifications of the spectrofluorometer.

Four analog signals, two photomultipler signals and two wavelength encoder outputs are connected to a multiplexer-A to D converter combination (DEC model AF01). Under program control, the computer can switch the multiplexer to admit any one of these voltages to the A to D converter where it is digitalized and from which it can be read into the computer. The computer applies instrumental and photophysical corrections to the data and stores it in the memory buffer until called up for display on the screen of the control terminal. A more complete description is available in the paper by Holland et al. (1973).

APPENDIX B

TABLE 5.--Pucks$_f$ plus glucose balanced salt solution.

Inorganic Salts	mg/liter
$CaCl_2 \cdot (2 H_2O)$	16.0
KCl	285.0
$MgSO_4 \cdot (7 H_2O)$	154.0
NaCl	7,400.0
$NaHCO_3$	1,200.0
$Na_2HPO_4 \cdot (7 H_2O)$	290.0
Other Components	
Glucose	1,100.0

(GIBCO)

APPENDIX C

TABLE 6.--Dilution corrected photometric data from iodination reactions.

Time (min)		PRL				IPO				KI			H_2O_2			
		2	4	6	8	12	14	16	18	20	24	26	28	30	32	58
	AB	.223	.230	.199	.227	.446	.440	.452	.436	.420	.443	.436	.448	.451	.446	.430
1	CO	6.0	6.1	6.5	6.1	11.8	11.5	11.9	11.7	10.5	10.3	10.6	8.5	7.1	7.0	6.8
	PQ	25.2	25.3	26.2	25.4	28.5	27.9	29.2	28.8	25.6	26.5	24.7	20.4	17.8	18.0	16.7
	AB	.418	.412	.410	.420	.763	.758	.771	.760	.755	.755	.760	.749	.750	.743	.745
2	CO	11.0	11.3	11.1	11.4	20.3	20.5	20.0	20.2	18.0	18.9	18.4	14.8	13.4	13.3	12.9
	PQ	27.2	28.0	27.0	27.4	31.6	32.0	30.7	30.9	27.3	29.5	28.1	23.4	20.5	20.6	20.0
	AB					.132	.130	.131	.128				.184	.150	.155	.152
3	CO					4.9	4.5	4.8	4.6				4.0	3.3	3.3	3.1
	PQ					14.3	14.3	14.8	14.2				9.9	9.1	8.8	9.2
	AB					.244	.225	.227	.227				.235	.240	.236	.228
4	CO					6.7	6.1	6.6	6.1				5.3	5.0	4.9	4.3
	PQ					12.6	12.8	12.0	12.7				9.0	8.5	8.4	8.0

AB = absorbance; CO = corrected fluorescence; PQ = partial quantum efficiency.
Vertical columns = minutes of reaction; vertical lines separate the additions of lactoperoxidase (LPO), potassium iodide (KI), and hydrogen peroxide (H_2O_2).

APPENDIX D

TABLE 7.--Prolactin-cell interactions at various cell and hormone concentrations (288∫∫348).

Initial CO (X)	PRL CO	Final CO - PRL CO (Exp)	Exp. - Control (Y)
17.5 µg/ml			
64 ± 1	16.1	43.9 ± 1	0.0 ± 2
141 ± 1	16.1	114.0 ± 1	-2.0 ± 2
225 ± 1	16.1	163.9 ± 1	2.9 ± 2
311 ± 1	16.1	227.9 ± 1	4.9 ± 2
424 ± 2	16.1	310.9 ± 2	5.0 ± 4
25 µg/ml			
48.4 ± 1	23.4	34.1 ± 1	2.1 ± 2
65.4 ± 1	23.4	50.4 ± 1	6.4 ± 2
89.8 ± 1	23.4	70.3 ± 1	7.3 ± 2
169.5 ± 1	23.4	127.6 ± 1	7.6 ± 2
183 ± 1	23.4	139.9 ± 1	9.9 ± 2
250 ± 1	23.4	194.6 ± 1	15.6 ± 2
302 ± 1.5	23.4	235.6 ± 1.5	18.6 ± 3
338 ± 1.5	24.0	263.0 ± 1.5	20.0 ± 3
430 ± 2	24.0	326.0 ± 2	20.0 ± 4
45 µg/ml			
92 ± 1	42.0	73 ± 1	9.0 ± 2
187 ± 1	42.0	145 ± 1	12.0 ± 2
248 ± 1	42.0	196 ± 1	18.0 ± 2
375 ± 1.5	42.0	309 ± 1.5	39.0 ± 3
434 ± 2	42.0	377 ± 2	65.0 ± 4

	diff. ± experimental variance*	diff. ± experimental variance**

All units are arbitrarily standardized against 10^{-5} M quinine sulfate in 0.1 M H_2SO_4.

Initial CO can be converted to cell concentration by use of the standard curve in the text.

*Experimental variance = variance of three sequential scans.

**Exp. var. = sum of variances from control and exp. data.

Control = CO of 2 ml of C$_8$11RAP cells + 0.25 ml of Pucks$_f$ + glucose.

Initial CO = CO of 2 ml of C$_8$11 RAP cells alone.

PRL CO = CO of PRL in 2.25 ml of Pucks$_f$ + glucose.

Exp. CO = CO of PRL - Cell mixture (2.25 ml) - PRL CO.

APPENDIX E

TABLE 8.--Dose response of prolactin-cell reactions.

Initial CO	PRL CO	[PRL] (X)	Final CO - PRL CO (Exp)	Exp. - Control (Y)
330	4.0	5.6	290 ± 1	2.0 ± 2
310	16.1	17.5	281 ± 1	5.0 ± 2
312	15.8	17.5	283.5 ± 1	4.5 ± 2
332	21.0	22.0	300.5 ± 1.5	12.5 ± 3
302	23.4	25.0	282.0 ± 1.5	18.6 ± 3
321	24.0	25.0	307.0 ± 1.5	19.0 ± 3
326	24.0	25.0	314.1 ± 1.5	22.0 ± 3
290	28.1	30.0	278.0 ± 1.5	32.0 ± 3
290	48.0	50.0	286.0 ± 2.5	38.2 ± 5
309	91.4	100.0	311.2 ± 2.5	46.6 ± 5
312 ± 15.2 (Mean ± S.D.)			diff. ± experimental variance	diff. ± experimental variance

Symbols as in Appendix D.

All units are arbitrary but standardized against 10^{-5} M quinine sulfate in 0.1 M H_2SO_4 (350$\int\int$450).

APPENDIX F

TABLE 9A.--Changes in light scattering of $C_8$11RAP cells due to dilution with buffer.

C.C.	1.4				1.9			2.9				5.6
I	8.9	10.9	19.1	25.5	30.1	37.0	50.0	52.1	75.5	107	131	169
F	8.5	9.6	17.6	24.3	26.5	33.0	37.5	36.2	51.5	75.2	96	119
Δ	0.4	1.3	1.5	1.2	3.6	4.0	12.5	15.9	24.0	31.8	35	50
I.V.	±1	±1	±1	±1	±1	±1	±2	±2	±3	±4	±5	±5

I = initial light scattering in arbitrary units.
F = light scattering following addition of 0.25 ml $Pucks_f$ + glucose.
Δ = I - F.
I.V. = instrumental variance of three sequential time scans.

TABLE 9B.--Changes in light scattering due to simultaneous dilution and addition of 40 μg/ml PRL.

C.C.	2.1		3.1		4.7	
I	41	60	77	109	133	167
I_{PRL}	2.6	2.6	2.6	2.6	2.6	2.6
F	32.2	35.2	58	75	92.7	108.4
Δ	8.8	24.8	29	34	38.3	58.6
I.V.	±2	±2	±3	±4	±5	±5

I = initial light scattering of cells.
I_{PRL} = light scattering of PRL in 2.25 and $Pucks_f$.
F = light scattering of cells following addition of 0.25 ml of buffer and 80 g PRL - I_{PRL}.
Δ = I - F.
I.V. = instrumental variation of three sequential scans.
C.C. = cell concentration x 10^6/ml.

APPENDIX G

Statistics

I. Mean and Standard Deviation (Dunn, 1964: 78-95)

$$\bar{x} = \frac{\Sigma x}{n} = \text{mean}$$

$$s = \sqrt{\frac{\Sigma x^2 - n\bar{x}^2}{n - 1}} = \text{standard deviation of the mean}$$

II. Students Tests for the Difference Between Means with Unknown Variance (Dunn, 1964: 78-95)

$$t = \frac{\bar{x}_1 - \bar{x}_2}{Sp\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{where } Sp = \sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1 + n_2 - 2}}$$

t = students distribution number of $n_1 + n_2 - 2$ degrees of freedom

III. Linear Regression (Sokal and Rohlf, 1969: 494-548)

$$\hat{y} = y + b\,(x-\bar{x})^2$$

$$b = \frac{\Sigma(x-\bar{x})\,(y-\bar{y})}{\Sigma(x-\bar{x})^2} = \text{regression coefficient}$$

$$r = \frac{\Sigma\,(x-\bar{x})\,(y-\bar{y})}{\sqrt{\Sigma\,(x-\bar{x})^2\;\Sigma\,(y-\bar{y})^2}} = \text{correlation coefficient}$$

$$S_r = \sqrt{1 - r^2/n-2} = \text{standard error of r}$$

n = sample number
Σ = summation
$\bar{y}$ = mean of individual y values

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