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ABSTRACT

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This study investigated the effects of a phosphodiesterase inhibitor, HL725 (Hoescht-Roussel Pharmaceutical, Inc.), believed to be selective for cAMP phosphodiesterase, on intraocular pressure and ciliary process cyclic nucleotide levels in rabbits. The albino rabbits used in this study demonstrated a reproducible circadian rhythm of IOP when housed under standard conditions with a 12:12 light:dark cycle. Topical applications of HL725, 0.1% and 1% in saline and 1% and 5% in BSS/-hydroxypropylmethylcellulose, caused small but significant decreases in IOP in the treated eye in comparison to the contralateral control eye. Intravitreal injections of HL725, resulting in calculated final intravitreal concentrations between 10^{-10} and 10^{-3} M, caused no change in IOP. Intravitreal injections of HL725 resulting in final intravitreal concentrations of 10^{-5} and 10^{-6} M potentiated the ocular hypotensive effect of topically applied isoproterenol 0.01%. HL725 increased ciliary process cAMP and cGMP over basal levels, suggesting that it was a nonselective inhibitor of cyclic nucleotide phosphodiesterases in ciliary processes in the concentration range studied.

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INTRODUCTION

A. Circadian Rhythms of IOP: An Indication of the Existence of a Regulatory Mechanism Controlling IOP

The observation of a circadian variation of intraocular pressure (IOP) in normal eyes and of larger fluctuations of IOP in glaucomatous eyes has led investigators to postulate the existence of regulatory mechanisms maintaining normal levels of IOP and malfunctions in some portion or portions of that regulatory mechanism occurring in glaucoma. A daily cyclic variation in human IOP was first noted in glaucomatous patients in 1898 by Sidler-Huguenin who reported tactile measurements of IOP with two pressure peaks daily, one before sleep and another shortly after waking. Several investigators [Maslenikow, 1905; Pissarello, 1915; Kollner, 1916; Thiel, 1925] later reported tonometric measurements demonstrating that glaucomatous eyes exhibited a pattern of a single maximal IOP in the early morning and a minimum pressure in the evening. Others [Langley and Swanljung, 1951; Macdonald, 1956; Hager, 1958] documented five types of diurnal variation curves: double peaking in the morning and again in the early evening, single peaking in the morning, single peaking in the early evening, varying sporadically or irregularly, and essentially flat. In all the studies the more common curve was that which peaked in the morning, fell in the afternoon, and had a lower peak in the early evening [Langley and Swanljung, 1951; Drance, 1960; Katavisto, 1964]. A more recent study measuring IOP frequently throughout the twenty-four hour period further

characterized the circadian cycle as having its minimum between two and four a.m. [Henkind et al., 1973]. Although several patterns of circadian variation could be found in glaucomatous patients, each individual's rhythm appeared to be constant unless the sleep-wake cycle was changed [Kollner, 1916; Hager, 1958].

Diurnal rhythms occur also in normal eyes but with smaller variations. In 1904 Maslenikow documented that the IOP of normal eyes exhibited a cyclic pattern with variations of IOP averaging 2 mm. Hg., significantly less than the fluctuations seen in the IOP of glaucomatous eyes. Later studies [Duke-Elder, 1952; Drance, 1960; deVenecia and Davis, 1963; Katavisto, 1964] recorded average diurnal variations of IOP for non-glaucomatous patients in the range of 3 to 7.5 mm. Hg.. Again the most common IOP curve had a large morning peak, a smaller evening peak, and a decline of IOP through the night. The comparison of normal and glaucomatous eyes revealed that larger fluctuations in IOP occurred in glaucoma, that IOP usually became more labile before becoming abnormally elevated, and that the diagnosis of ocular hypertension could be missed if only one measurement of IOP was taken during the day [Drance, 1960; Katavisto, 1964]. Physicians were cautioned to measure IOP more than once daily in patients having borderline tensions and to take into account the circadian pattern when treating ocular hypertension.

Circadian IOP rhythms have been detected also in rabbits, crepuscular mammals [Thompson and Worden, 1956] used frequently in ophthalmic research. Rabbits kept for eight days in a facility where the light

dark schedule corresponded to the sunrise-sunset schedule were transferred to a lab with constant artificial lighting for the 24-hour period of IOP measurements [Vareilles et al., 1977a]. They exhibited a pattern of low IOP at night, a sharp rise in IOP between 6 and 8 a.m., maintenance of a high IOP throughout the day, and a decline in the early evening. A second group of rabbits was kept for seven to ten days in a room with a light/dark schedule of 7 a.m. to 5:30 p.m. light/ 5:30 p.m. to 7 a.m. dark, but was moved to a laboratory with a greater light/dark ratio (7:30 a.m. to 11:30 p.m. light/ 11:30 p.m. to 7:30 a.m. dark) for the measurement period [Katz et al., 1975]. A third group entrained on a 5 a.m. to 5 p.m. light/ 5 p.m. to 5 a.m. dark schedule was transferred to a measurement laboratory where the artificial lighting conditions were not specified but the daylight source varied according to the season [Bar-Ilan, 1984]. The second group had two diurnal peaks of IOP, a smaller one between 8 and 11 a.m. and a larger one between 4 and 7 p.m., and a minimum recorded between 7 and 9 p.m. The third group also exhibited two daily peaks, one small peak at 3 p.m. and a large peak at 10 p.m., with IOP falling between 10 p.m. and midnight (no measurments were recorded between midnight and 9 a.m.). Although a general cycle of high diurnal IOP and low nocturnal IOP was seen in all three groups these three studies reported three different circadian rhythms. The three patterns may have differed in the timing of peaks and troughs and in the shapes of the curves possibly because of the differences in the artificial light-dark schedules and sunrise-sunset timing as well as animal behavior patterns. The investigators pointed to the necessity of careful consideration of IOP patterns and documentation of control values in utilizing a rabbit mod-

el, but none apparently realized the effect that changes in lighting conditions of the measurement environment might have had on the rabbits' circadian rhythms.

Taking into account the importance of entrainment and maintenance of a controlled environment for the preservation of circadian rhythms, a group of researchers designed an experimental protocol which could provide a useful rabbit model for the investigation of circadian rhythms of IOP [Rowland et al., 1981]. Measurements were taken without changing the lighting conditions by utilizing a far-red range light during periods of darkness. The pattern of daily IOP variation was opposite to that observed in the three previous studies. Highest IOPs were seen during the night and lowest IOPs during the day, with sharp rises and falls in IOP occurring at the beginning of darkness and light, respectively. The authors demonstrated that the rabbits' circadian rhythm was preserved in constant darkness but eliminated by constant light, in which the rabbits' IOP pattern became almost flat at a mean IOP level. They proposed that a leveling off of IOP at a midrange pressure in constant light implied that the mechanisms controlling IOP, entrained by the light-dark schedule, acted to increase IOP in darkness and decrease it in light. A recent study [Gregory et al., 1985] of the circadian rhythm of rabbits, performed under similar experimental conditions employing a well-controlled environment and a long wavelength red light, confirmed that IOP is higher in dark and lower in light and that the pattern continues in constant dark. By reversing the light-dark cycle and documenting a reversal of the IOP cycle they demonstrated that the light-dark schedule was the cue necessary for entrainment

of the IOP rhythm. They remarked upon the prolonged persistence of the IOP pattern in constant dark as an indication that the rhythm was truly circadian with a period close to 24 hours. Both of these investigations, employing conditions necessary for preservation of circadian rhythms, indicated that rabbit IOP circadian pattern, although exemplifying a light-dark cycle reverse to that reported for humans, could be utilized as a reproducible animal model for the study of factors affecting and controlling the human circadian rhythm of IOP.

Seasonal as well as diurnal variations in IOP were observed in rabbits [Vareilles et al., 1977; Bar-Ilan, 1984]. Normal and glaucomatous patients also were reported to have seasonal IOP fluctuations. Seasonal variation in human IOP, with highest IOPs in January and February and lowest IOPs in July and August [Blumenthal et al., 1970; Bengtsson, 1972], were noted to be similar to seasonal changes in other physiologic parameters such as heart rate, basal metabolic rate, and adrenal cortical activity (all observed to have the reverse seasonal pattern with values higher in summer than in winter). The analogy between seasonal and circadian IOP variations and cyclic changes in other physiologic values incited an attempt to explain the cause of these fluctuations by correlating IOP changes with variations in hormonal or other biochemical substances in the plasma or CNS. One group of investigators [Schmerl and Steinberg, 1955] extracted from the CSF two substances which they named hyperpiesin and miopiesin. They demonstrated that hyperpiesin caused an increase in IOP, miopiesin caused a decrease in IOP, and that the amount of hyperpiesin and miopiesin in the CSF was related to light and dark conditions. In man a higher CSF concentra-

tion of miopiesin occurred in light and a higher CSF concentration of hyperpiesin occurred in dark. In rabbits, the correlation between conditions of light and dark and CSF concentrations of miopiesin and hyperpiesin was reversed. From their results they postulated that the anterior pituitary secreted hyperpiesin, the posterior pituitary stored hyperpiesin and transformed it to miopiesin via a catalyst (activated by light and heat), that hyperpiesin caused an increased IOP via parasympathetically mediated vasodilation and miopiesin caused a decreased IOP via sympathetically mediated vasoconstriction, and that under normal circumstances the CSF contained both substances in amounts which provided a physiologic balance and a normal level of IOP. Another group of investigators [Weitzman et al., 1975] studied the relationship between plasma cortisol concentrations (known to be preceded by changes in plasma ACTH concentrations) and IOP. (This relationship was of particular interest because of the known parallelism between circadian curves of plasma catecholamine and cortisol concentrations [implying that adrenomedullary and adrenocortical activity might both be influenced by ACTH secretion circadian rhythms] and because of information implicating neural transmitter substances in the regulation of other endocrine functions [Eleftheriou, 1974].) They demonstrated a similarity between the circadian patterns of the two levels, with the plasma cortisol and the IOP 24-hour curves having a phase difference of three hours between the mean nadir and zenith, the cortisol curve preceding the IOP curve. The dexamethasone suppression test ablated the cortisol but not the IOP curve, indicating that the relationship between the two curves was correlative, not causative. (The lack of a causative relationship is not surprising in light of the present knowledge that IOP is affected by

the light-entrained [Rowland et al., 1981; Gregory et al., 1985] "Y" pacemaker and plasma cortisol by the "X" pacemaker [Moore-Ede et al., 1982]). Evidence from these two studies, and the analogy between circadian rhythms of IOP and of many other physiologic functions which were known to be controlled by the hypothalamus, suggested but did not prove that neural and humoral control of IOP might exist.

B. Circadian Rhythms of IOP: Studies of Aqueous Humor Dynamics

Ophthalmologists have attempted to explain not only the possible central and hormonal causes of circadian fluctuations in IOP but also the importance of changes in aqueous humor dynamics. Although Stepanik [1954] found that outflow resistance was highest when the maximum IOP occurred during the diurnal rhythm and Drance saw a diurnal variation of outflow facility (C) in glaucomatous patients, Grant [1955] reported an unchanging outflow facility throughout the circadian cycle and subsequent studies [Newell and Krill, 1964; Radnot et al., 1970] were unable to confirm a definite or statistically significant relationship between the variations of IOP and of outflow facility. In contrast, investigations of aqueous humor formation (AHF) have shown that changes of AHF correspond to changes in IOP in the circadian rhythm [Grant, 1955; Ericson, 1958; Reiss et al., 1984]. The results of these few experiments, along with the report of the constancy of episcleral venous pressure (EVP) [Linner, 1956], suggest that of the parameters: AHF, C, and EVP, aqueous humor formation plays the most important role in circadian variations of IOP. A circadian rhythm of uveoscleral outflow, another

component of aqueous humor dynamics [Bill, 1965 and 1970] which accounts for only a small percentage of total outflow in man [Jocson and Sears, 1971], has not been shown.

Recent studies of AHF by fluorescein clearance techniques have resulted in possible explanations of the role of AHF in circadian IOP changes and for the neurohumoral regulation of circadian IOP variations. Measurements of AHF between 8 a.m. and 1 p.m., noon and 5 p.m., and midnight and 5 a.m., showed rates of 3.1, 2.4, and 1.6 microl./min., respectively [Brubaker et al., 1984; Reiss et al., 1984]. AHF during the morning hours was twice as rapid as the flow rate at night during sleep; an intermediate rate of flow was seen in the afternoon hours. Subjects who were sleep-deprived demonstrated a nighttime AHF of 2.3 microl./min., higher than the AHF rate (1.6 microl./min.) of subjects whose sleep was undisturbed. The AHF of sleep-deprived patients may have been higher than the normal nighttime AHF because sleep is required for "maximum suppression of aqueous flow" [Reiss et al., 1984], or because the subjects' regular circadian pattern of AHF was changed by exposure to light or the nocturnal pattern was altered by activity and eating during the period of time of normally undisturbed sleep. The authors noted that the AHF rate during sleep was comparable to the AHF rate which they had documented in patients treated with carbonic anhydrase inhibitors and beta-blockers. Shortly thereafter it was found that during the day timolol and acetazolamide decreased normal daytime AHF 30 and 20%, respectively, while epinephrine increased daytime AHF 15%. During the night neither timolol nor acetazolamide had any significant effect on AHF, while epinephrine increased the

normal flow of AHF during sleep by 47% [Topper and Brubaker, 1985a and b]. During the day in the supine position subjects had an AHF 12% less than the normal daytime AHF. Based upon these results and the knowledge that plasma epinephrine concentrations are reduced during sleep and in the supine position, the authors postulated that the circadian rhythm of AHF is caused by normally circulating epinephrine stimulating aqueous humor production and that the mechanism of action of timolol and other beta-blockers (a molecular mechanism which has not been elucidated despite many hypotheses proposed to date) was to block AHF stimulation by epinephrine or another endogenous mediator.

C. A Regulatory Mechanism for Control of IOP: The Hypothesis of Centrally Mediated Control

The hypothesis that epinephrine or another catecholamine is involved in the control of IOP is not a new one. A role for the sympathetic nervous system in IOP control was postulated as early as the 1860's when cervical sympathetic ganglionectomy [Wegner, 1866; Hertel, 1900] and cervical sympathetic nerve stimulation [vonHippel and Gruenhagen, 1870] resulted in a decrease in IOP. The use of adrenaline (epinephrine) for the treatment of glaucomatous patients was based on the belief that elevated IOP in glaucoma resulted from a sympathetic neurosis of the uveal vessels causing vascular atony and increased blood volume [Hamburger, 1914 and 1923]. Darier [1900] gave subconjunctival injections and Hamburger [1923] gave topical applications of

epinephrine to patients with glaucoma. Thiel [1931] believed that a disturbance of regulation of intraocular circulation and therefore blood-tissue fluid exchange was one of the causes of glaucoma. Duke-Elder [1932] pointed to the presence of sympathetic fibers in the uveal tract as evidence for control of intraocular vessel tone by the sympathetic nervous system. During that period of time many ophthalmologists believed that an abnormality in the function of the sympathetic fibers in the eye was responsible for IOP elevations [Elwyn, 1938].

Based upon these authors' postulates, the observation that diurnal rhythms of IOP were comparable to changes in other "vegetative functions," and the evidence for regulation of other normal physiologic values by a central mechanism, Elwyn [1938] formulated an hypothesis for an IOP regulatory mechanism analogous to that of other physiologic functions. This consisted of an hypothalamic control center, neural and humoral "channels of influence," and an effector organ (in this case, the eye). According to his hypothesis the effects of the neural "channels of influence" would be carried from the hypothalamus through the brainstem and spinal cord and thence through the sympathetic and parasympathetic nervous systems to the fibers ending in the eye. The humoral regulation would occur by the hypothalamus exerting effects on the hypophysis to secrete hormones into the circulation from whence they would be carried to the eye. Normal IOP would be maintained by impulses sent from theoretical pressure sensors in the eye (whenever IOP wandered outside a normal range) to the control center from which, in response, hormones or nervous impulses would be delivered back to the eye to reestablish a normal IOP. Elwyn furthermore proposed that

some abnormality in this regulatory mechanism was the cause of glaucoma. He compared the early increasing lability of IOP and subsequent elevated IOP with the earlier large fluctuations and subsequent elevations of blood pressure and blood glucose. He believed that glaucoma, as well as systemic hypertension and diabetes, resulted from changes in the regulatory mechanism which caused first increasingly variable and later abnormally high IOP, blood pressure, and blood glucose.

Clinical observations of glaucoma patients led Hess, in 1946, and other physicians [Zondek and Wolfsohn, 1947; Magitot, 1947] to hypothesize the existence of similar IOP regulatory mechanisms with the diencephalon as the center of control. Attempts to locate this center and to define its influence on IOP were made by stimulating areas of the diencephalon and hypothalamus of rabbits and cats [Schmerl and Steinberg, 1950; Nagai et al., 1951; Takagi, 1952; Weinstein, 1954; vonSallman and Lowenstein, 1955; Gloster and Greaves, 1957; Duke-Elder, 1957]. None of these investigators identified conclusively a region of the CNS which appeared to exert prolonged influences on IOP separate from its effects on blood pressure, extraocular muscle contraction, serum osmolarity, or other factors.

Researchers continued to hypothesize that the hypothalamus might be the controlling site for changes in IOP [Waitzman, 1971] and continued to search for some form of hypothalamic control on IOP. In the attempts to corroborate the existence of an osmotic central regulatory mechanism for IOP control, mediated by the hypothalamic osmoreceptors and "efferent" fibers of the optic nerve, studies were performed on pa-

tients with unilateral optic nerve lesions and on animals after unilateral optic nerve transection [Riise and Simonsen, 1969; Krupin et al., 1970]. Even though optic nerve efferent fibers had been traced from the anterior hypothalamus [Sacks and Lindenburg, 1969] and the rabbit and human eyes with transected optic nerves did show altered responses to osmotic agents in comparison to eyes with intact optic nerves, the results of these studies only suggested, did not prove, that hypothalamic osmoreceptors might be involved in IOP control.

Manipulation of the CNS by direct stimulation of the hypothalamus and by induction of osmotic changes failed to locate an IOP control center in the CNS. Attempts to demonstrate a pressure sensor in the eye or a regulatory feedback mechanism were equally unsuccessful. Intraocular infusion experiments, in which IOP was varied at the same time that IOP and C were monitored, did not indicate the presence of any apparent feedback mechanism for the maintenance of a normal level of IOP [Sears, 1960]. Pseudofacility, the pressure-sensitive portion of AHF detected as a facility by tonographic measurements [Barany, 1963], was believed by some to be a local IOP regulatory mechanism acting to diminish IOP increases by decreasing ultrafiltration (and therefore AHF) whenever IOP increased [Barany, 1963; Bill and Barany, 1966; Kupfer and Sanderson, 1968; Bill, 1968; Brubaker, 1970]. As the suppressability of aqueous humor formation, pseudofacility was postulated also to be a manifestation of a feedback and central regulatory mechanism for IOP control [Bill,1969]. Ultrafiltration appears to be unimportant in AHF [Caprioli, in press], and pseudofacility may not be an extant phenomenon [Moses, 1985].

The inability to demonstrate prolonged IOP changes by manipulation of the CNS does not preclude the existence of an IOP central regulatory mechanism. In fact, the IOP circadian rhythm, known in rabbits to be entrained by the zeitgeber light [Rowland et al., 1981; Gregory et al., 1985], provides evidence for a control center located in the CNS. In mammals the suprachiasmatic nuclei (SCN) have been shown to be the site of the primary pacemaker associated with the light-dark cycle [Moore-Ede et al., 1982 & 1983]. This pacemaker appears to be entrained by light signals transmitted through the retinohypothalamic tract (RHT) and to control circadian rhythms of skin temperature, urine calcium excretion, plasma growth hormone levels, rest and activity, sleep and waking, and drinking. In humans a similar cluster of neurons, located more laterally in the anterior ventral hypothalamus above the third ventricle, is believed to contain the light-dark cycle associated pacemaker [Moore-Ede et al., 1982 & 1983], and a retinosuprachiasmatic pathway may be the route of transmission of light signals entraining the central circadian pacemaker [Sadun et al., 1984]. The demonstration of light as the zeitgeber for entrainment of the IOP circadian rhythm [Rowland et al., 1981; Gregory et al., 1985] indicates that a circadian timing system with a pacemaker located in the CNS exerts control of IOP. The IOP increases of the circadian cycle in rabbits appear to be mediated by adrenergic innervation [Gregory et al., 1985].

D. A Regulatory Mechanism for Control of IOP: Indications of Hormonal Influences on IOP

There is evidence substantiating the existence of Elwyn's humoral

"channels of influence" [1938] as well as the control center located in the CNS. The possibility of hormonal control of IOP was suggested by the clinical observation of low IOP in patients with pregnancy and myotonic dystrophy, conditions associated with elevated levels of circulating hCG and FSH, respectively [Imre, 1922; DeGrosz, 1937; Horven and Gjonnaess, 1974; Brand, 1955; Burian and Burns, 1967]. Numerous other studies correlating ocular hypotensive and hypertensive states with physiologic hormonal changes and with diseases characterized by abnormal hormone levels [Kass and Sears, 1977] suggested a role for various glycoprotein and steroid hormones in the regulation of IOP. Significant decreases of IOP in menopausal glaucomatous patients [Niebroj et al., 1971], in infertile females [Rubin, 1985], and in rabbits [Gierkowa et al., 1976; Rubin, 1985] occurred when hCG was administered intramuscularly. Intravitreal injections of the glycoprotein hormones FSH, LH, hCG, and TSH, but not progesterone, caused decreases in IOP of rabbits with a potency order: FSH = LH > TSH > LH [Sears and Mead, 1983]. These investigations confirmed the influence of glycoprotein hormones on IOP and the possibility of hormonal regulation of IOP.

E. A Regulatory Mechanism for Control of IOP: The Role of the Adrenergic Nervous System

Sympathetic control of IOP was postulated as early as the nineteenth century [Wegner, 1866; vonHippel and Gruenhagen, 1870]. Since that time, years of experience with adrenergic agents in treating glaucoma and in investigating their effects on components of aqueous

humor dynamics have led to further conjectures of the role of the sympathetic nervous system in local neural IOP control. Several factors have caused considerable confusion in interpreting results of the innumerable investigations of adrenergic agents' effects on IOP. It has been difficult to separate the complex interactions between intraocular structures which bring about changes in individual components of aqueous humor dynamics and result in a net change in IOP. It has been even more difficult to draw conclusions from (or to make comparisons between) experiments which differ in almost every aspect of design, including routes of drug administration (and therefore pharmacodynamics), techniques of aqueous humor dynamics measurement, animal species (and therefore intraocular structures and adrenergic receptors), and timing of measurements [Mishima, 1982; Sears, 1984]. Within this text a comprehensive review will not be attempted but instead an historical perspective and overview of the resulting theories will be presented.

In the 1950's Goldmann [1951] and Weekers [1955] demonstrated that adrenaline (epinephrine) lowered IOP and AHF in humans without affecting outflow facility. In contrast, the results obtained from tonography and long term follow-up of glaucomatous patients treated with epinephrine suggested that it reduced IOP by increasing C. Garner [1959] first noticed in a few patients that outflow facility was increased, and other investigators subsequently reported short and long term increases in C [Becker, 1961; Ballantine, 1961; Galin et al., 1966; Kronfeld, 1971]. The effect of epinephrine in humans was described then as having three components: (1) an early decrease in AHF, (2) an early increase in C, and (3) long term progressively increasing C

[Sears, 1966]. The early decrease in AHF was presumed to be secondary to decreased blood flow to the ciliary processes [Sears, 1966], possibly by alpha-adrenergically mediated vasoconstriction of uveal vessels [Mishima, 1982], although recent studies suggest that decreased blood flow to the iris-ciliary body is not short term [Caprioli et al., 1984].

Assuming episcleral venous pressure (EVP) is not affected by beta-adrenergic drugs (and is approximately 9 mm.Hg.) [Mishima, 1982] and that uveoscleral outflow is only a small percentage of total outflow in humans [Jocson and Sears, 1971], changes in IOP can be attributed to the sum of changes in outflow resistance (R) and in aqueous humor formation (AHF). In order to interpret the effects of adrenergic drugs on IOP one can calculate reduction ratios of outflow pressure and of outflow resistance from human experimental data [Mishima, 1982]. From the relationship:

$$\{ \text{deltaIOP}/(\text{IOP}-\text{EVP}) \} = \{ \text{deltaR}/R + \text{deltaAHF}/\text{AHF} \},$$

[where: (1) outflow pressure = (IOP-EVP);(2) $R = 1/C$; (3) delta IOP/(IOP-EVP) = reduction ratio of outflow pressure; and (4) deltaR/R = reduction ratio of outflow resistance or reciprocal of reduction ratio of outflow facility], one can see that a high correlation between the reduction ratios of outflow pressure and of outflow resistance would indicate that changes in outflow resistance (the reciprocal of C) primarily are responsible for changes in IOP. A poor correlation between outflow pressure and outflow resistance reduction ratios would

indicate a significant role for AHF in causing the IOP changes. Analysis of data from clinical investigations of epinephrine's early and late hypotensive effects showed a high correlation between the outflow pressure and outflow resistance reduction ratios for almost all studies. Short and long term increases in C appear therefore to be responsible for the short and long term hypotensive effects of epinephrine [Mishima, 1982]. The early increase in C may be caused by alpha- and beta-adrenergic effects, and the long term progressively increasing C is perhaps related to the activation of lysosomal hyaluronidase and therefore to metabolism of trabecular meshwork glycosaminoglycans decreasing outflow [Hayasaka and Sears, 1978].

Evidence for the acute alpha-adrenergic effect of epinephrine on C was obtained from a series of cervical sympathetic ganglionectomy experiments on rabbits. Early ganglionectomy studies demonstrated a decrease in IOP, and measurements of C along with IOP suggested that a decrease in AHF caused the prolonged decrease in IOP seen hours to days after ganglionectomy [Linner and Prijot, 1955; Lieb et al., 1958]. Langham and Taylor [1959 and 1960] and Sears and Barany [1960] demonstrated on the contrary that the decrease in IOP was secondary to an increase in C and that the effect was alpha-adrenergically mediated [Sears and Barany, 1960]. Further investigations indicated that postganglionic section resulted in norepinephrine release from the iris into the aqueous humor where it caused an alpha-mediated increase in outflow, and that the increased C and decreased IOP seen in eyes with an intact sympathetic system was short term and slight because of the inactivation of norepinephrine via uptake and binding by the iris [Bara-

ny, 1962; Rosser and Sears, 1963; Eakins, 1963; Eakins and Eakins, 1964; Sears and Sherk, 1964; Sears et al., 1966; Sears and Gillis, 1967; Rosser and Sears, 1968]. The short term increase in outflow facility caused by epinephrine in humans and rabbits was, therefore, at least partially mediated by alpha-adrenergic effects on the outflow channels [Sears, 1975].

Conflicting results have been obtained for the possible role of beta-mediated effects on outflow in rabbits and man. In the rabbit a series of experiments [Neufeld et al., 1972; Neufeld et al., 1973; Neufeld and Sears, 1974; Neufeld et al., 1975] suggested that epinephrine affected the outflow channels and decreased IOP by an increase in the aqueous humor cyclic 3',5'-adenylmonophosphate (cAMP) concentration, probably via a beta-receptor mediated mechanism [Sears, 1975]. A later study showing blockade by timolol of epinephrine's increase in aqueous humor cAMP concentration but not of epinephrine's hypotensive response [Boas et al., 1981] suggested, however, that the relationship between increased aqueous humor cAMP concentration and decreased IOP was not a causal one. Other investigations have provided evidence both for and against beta-adrenergic mediation of augmented outflow facility in the rabbit, and the issue has yet to be resolved [Mishima, 1982; Sears, 1984]. Experiments conducted in humans provided equally conflicting results concerning the possible role of the beta-receptor in increasing C [Mishima, 1982]. Epinephrine appears to increase uveoscleral outflow in humans [Townsend and Brubaker, 1980], and this effect may be secondary to beta-stimulated relaxation of the ciliary muscle [Townsend and Brubaker, 1980; Mishima, 1982]. Although

both alpha- and beta-receptor mechanisms may be involved in epinephrine's effects on C, the only conclusive evidence thus far indicates that the moderate early increases in C are caused by alpha-adrenergic stimulation [Sears, 1984; Caprioli, 1985].

Investigations of epinephrine's effects on AHF have provided results as conflicting as those on the role of the beta-receptor in outflow mechanisms. Since Goldmann's demonstration of epinephrine's ability to decrease AHF in man in 1951, numerous determinations of AHF have been made in rabbits and in man. Fluorophotometric techniques using topical and systemic administration of fluorescein have been utilized. Some investigators have cannulated the anterior chamber, possibly resulting in slight blood-aqueous barrier breakdown as demonstrated by slightly increased aqueous humor protein concentrations [Mishima, 1982]. As many studies have demonstrated decreased AHF [Goldmann, 1951; Weekers, 1955; Gaasterland et al., 1973; Takase, 1976; Nagataki, 1977; Green and Padgett, 1979; Araie and Takase, 1981; Miichi and Nagataki, 1982] as have shown increased AHF [Townsend and Brubaker, 1980; Nagataki and Brubaker, 1981; Schenker et al., 1981; Thomas and Epstein, 1981; Lee et al., 1983; Topper and Brubaker, 1985a and b] after epinephrine treatment of humans and rabbits. The different results obtained may be a reflection of the differences in experimental design (animal species used, timing of measurements, dosage and route of administration) as well as the limitations and sources of error of each fluorometric method [Mishima, 1982; Sears, 1984]. After a review of the literature examining the techniques and timing of measurements, several authors have concluded that epinephrine's effects on AHF probably include an early increase followed by a prolonged decrease in AHF [Mishi-

ma, 1977; Townsend and Brubaker, 1980; Sears, 1984]. It is apparent, however, that more carefully planned and extensive studies need to be done to characterize the time sequence of its effects (short and long term components as well as variation at different periods of time in the circadian rhythm) and the quality of its effects (small or large, hypotensive or hypertensive) and to clarify the alpha- and beta-receptor mechanisms involved [Mishima, 1982].

Other explanations have been proposed for the mechanism of action of epinephrine. One theory, based upon observations of a decrease in epinephrine's hypotensive effectiveness caused by indomethacin, suggests that epinephrine's IOP lowering ability may be mediated partially by prostaglandins or other cyclooxygenase products [Bhattacherjee and Hammond, 1977; Camras et al., 1985]. Another postulation was that epinephrine's delayed effects were caused by beta-adrenergic desensitization. Neufeld et al. [1978] documented a decrease in iris-ciliary body beta-receptors following repeated treatment of rabbits with epinephrine. Mittag and Tormay [1981] showed that a few doses of epinephrine could cause uncoupling of adenyl cyclase (AC) from its activating system by decreasing the guanyl nucleotide regulatory protein activity. Bartels et al. [1983] compared the IOP and beta-receptor population effects caused by epinephrine and timolol and concluded that epinephrine has two IOP-lowering effects: an early beta-agonism and a late beta-adrenergic desensitization. The late beta-adrenergic desensitization was hypothesized to cause a decrease in AHF in a manner similar to the AHF decline caused by the beta-blocker timolol [Mittag and Tormay, 1981; Bartels et al., 1983].

Information on the effects of norepinephrine, a mixed alpha- and beta-adrenergic agonist with predominantly alpha effects, was derived not from treatment of glaucomatous patients but from a series of cervical sympathetic ganglionectomy experiments. As previously outlined, norepinephrine was shown to be stored in adrenergic nerve terminals located primarily in the iris and to be released by degenerating nerve terminals shortly after postganglionic sympathetomy, causing an alpha-mediated increase in outflow facility. In an eye with intact sympathetic innervation the iris functions to inactivate norepinephrine by uptake and binding [Sears, 1975].

Despite its known ocular hypotensive effect, isoproterenol, an adrenergic agonist with primarily beta effects, has been studied less extensively than epinephrine because of the concern for its primary side effect, systemic tachycardia. When Weekers [1955] investigated a group of sympathomimetic amines along with adrenaline (epinephrine) he concluded that adrenaline lowered IOP by reducing AHF via a sympathetic stimulatory effect independent of the vasoconstriction. He based his conclusion on tonographic demonstration of unaltered C, fluorometric evidence for decreased AHF, clinical observation of short term conjunctival vasoconstriction in contrast to longer term tension reduction, and experimental documentation of isoproterenol's tension-lowering effect (similar to adrenaline's) without adrenaline's associated vasoconstriction and mydriasis. He theorized therefore that isoproterenol must lower IOP via a sympathetically stimulated mechanism causing a decrease in AHF. Several authors supported the conjecture that isoproterenol reduced IOP by reducing AHF with the demonstration of lowered IOP

and unaltered C [Eakins, 1963; Ross and Drance, 1970; Langham et al., 1971; Gaasterland et al., 1973] as well as decreased AHF [Gaasterland et al., 1973; Takase, 1976]. Although some investigators have shown that isoproterenol may slightly increase C [Eakins and Ryan, 1964; Bonomi, 1964; Gnadinger and Barany, 1964; Lorenzetti, 1971] (especially at higher concentrations where it could exert mild alpha effects) its primary role appears to be in decreasing AHF, as further supported by its ability to decrease IOP in rabbits with complete outflow obstruction [Bonomi et al., 1964]. A review of studies conducted in humans, comparing the reduction ratios of outflow pressure and outflow resistance, also supports the contention that isoproterenol reduces IOP without increasing C [Mishima, 1982]. Unfortunately isoproterenol's clinical usefulness has been limited not only by its toxic effects of systemic tachycardia and local hyperemia [Sears, 1982] but also by its decreased effectiveness when used chronically [Ross and Drance, 1970].

The clinical and experimental work with epinephrine, norepinephrine, and isoproterenol demonstrated that adrenergic agonism may lower IOP through effects on components of aqueous humor dynamics, including inflow, conventional outflow, and uveoscleral outflow, although EVP apparently is not changed at least by beta-adrenergic agonists [Mishima, 1982]. Early identification of adrenergic nerves in the ocular tissues, including the chamber angle and ciliary processes [Amsler, 1955; Holland et al., 1957; Laties and Jacobowitz, 1964; Ehinger, 1966], added support to the belief in adrenergic regulation of IOP, but later investigations failed to clarify the mechanistic details of the role of the adrenergic agonists in controlling IOP. It is difficult to

define specific effects or to locate sites of action of adrenergic agents based only on pharmacologic and physiologic studies in humans and other animals, because of complexities of ocular tissue interactions and pharmacodynamics, species differences of ocular tissue adrenergic receptors, and the limits of available measurement techniques [Mishima, 1982; Sears, 1985].

The issue of the adrenergic nervous system's role in controlling IOP became more enigmatic after discovery of the ocular hypotensive effects of beta-blockers. In 1960 Sears and Barany demonstrated that dichloroisoproterenol, the first known beta-blocker, increased outflow facility. The cause of its effect on C and IOP became uncertain, however, when it was discovered to have intrinsic sympathomimetic activity. In 1967 Phillips et al. noted a lowered IOP in patients treated with intravenously administered propranolol, a beta-blocker with membrane stabilizing activity and no partial agonist activity. Subsequently numerous studies confirmed that propranolol, when administered intravenously, orally, or topically, caused a decrease in IOP although its side effects of ocular discomfort and local anesthesia prohibited its topical use [Wettrell, 1977]. Propranolol and other beta-blockers, including the cardioselective (beta $_1$) beta-blockers, have been shown to decrease IOP in humans as well as rabbits [Boger, 1979; Lotti et al. 1984]. Beta-blockers have had greater and more consistent hypotensive action in hypertensive than normal rabbits and in general have more pronounced effects on IOP in humans than in rabbits [Lotti et al., 1984]. Beta-blockers reduce IOP by decreasing AHF [Takats et al., 1972; Yablonski et al., 1978; Coakes and Brubaker, 1978; Reiss and

Brubaker, 1983; Lotti et al., 1984; Araie and Takase, 1985].

Of the many beta-blockers in existence timolol, a nonselective (beta$_1$ and beta$_2$) beta-blocker without intrinsic sympathomimetic activity or membrane stabilizing activity, has been employed most. It has been shown to (1) lower IOP in normal [Katz et al, 1976] and in glaucomatous [Zimmerman and Kaufman, 1977] eyes, (2) be more effective in lowering IOP than epinephrine or pilocarpine [Zimmerman and Boger, 1979], (3) lower IOP in the contralateral eye when applied topically [Lotti et al., 1984], (4) bind reversibly to ocular pigment thereby prolonging its availability to act in the eye [Bartels et al., 1983], and (5) have a potency eleven times that of propranolol [Takase and Araie, 1980]. Its clinical attractiveness is only minimally diminished by "short-term escape" and "long-term drift" [Zimmerman and Boger, 1979; Boger, 1983]. Some authors have attributed timolol's decreased effectiveness in the short term to the well-known phenomenon of beta-receptor density modulation as a self-controlling mechanism in the presence of beta-agonists or antagonists [Boger, 1983]. Indeed, an increase in beta-receptors in the cornea and the iris-ciliary body following prolonged treatment with timolol has been documented, but the tissues did not appear to be able to synthesize more cAMP (when stimulated by epinephrine) despite increased receptor density [Neufeld et al., 1978]. The causes of the short-term escape as well as the long-term drift remain unknown [Caprioli, 1985], and despite these characteristics it is one of the most useful ocular hypotensive agents available today.

Timolol acts, as do other beta-blockers, by lowering AHF [Yablonski et al., 1978; Coakes and Brubaker, 1978; Lotti et al., 1984; Topper and Brubaker, 1985]. How can the paradox of beta-agonists and antagonists both resulting in lowered AHF [Wettrell, 1977] be explained? Some investigators believe that timolol acts by beta-blockade alone to decrease IOP [Liu, 1983], although this theory is dependent on the demonstration of existence of endogenous adrenergic tone (from circulating epinephrine or another catecholamine) keeping AHF high in order for beta-blockade to cause a decreased AHF [Neufeld et al., 1983; Topper and Brubaker, 1985]. Others have documented more evidence arguing against timolol acting to lower AHF and IOP by beta-blockade: (1) higher concentrations of timolol are needed to lower IOP than to block beta-receptors [Vareilles et al., 1977b]; (2) d-timolol, a stereoisomer of timolol with significantly less beta-blocking activity than l-timolol in the eye [Liu et al., 1983] as well as other tissues, significantly lowers IOP in patients with ocular hypertension [Keates and Stone, 1984]; and (3) there is no correlation between the beta-blockers' hypotensive effectiveness, its antagonism of isoproterenol's hypotensive effect, its antagonism of isoproterenol's adenyl cyclase stimulation, and ciliary process beta-receptor binding and blocking potencies [Gregory et al., 1981; Schmitt et al., 1981; Sears and Mead, 1983; Lotti et al., 1984]. These authors have proposed that beta-receptors in the eye may be different from other systemic beta-receptors [Schmitt et al., 1981] or that timolol acts in the eye by another mechanism than beta-blockade [Sears and Mead, 1983]. Although it is unlikely that beta-receptors in the eye differ from those in the rest of the body [Sears and Mead, 1983] it is possible that ocular tissues respond differently to beta

blockers than do other tissues, as suggested by the contrast between the decreased ocular hypotensive effects and the increased systemic hypotensive effects associated with prolonged usage of beta-blockers [Boger et al., 1983]. Some beta-blockers prevent the activation of phosphodiesterase by calmodulin and of guanyl cyclase by nitroso compounds in the heart [Kudo & Nazawa, 1985]. It is possible that in the eye as in the heart beta-antagonists act not by blocking beta-receptors but by affecting a cellular enzyme such as a phosphodiesterase or guanyl cyclase.

F. A Regulatory Mechanism for Control of IOP: The Potential Role of the Adenyl Cyclase-Receptor Complex

What then are the possible regulatory mechanisms, central neural, neurohumoral, or hormonal, which could control IOP and result in the large fluctuations of IOP seen in the circadian rhythm? Light entrainment of the IOP circadian rhythm pointed to the SCN as the centrally-located pacemaker of the circadian system affecting IOP. Neurohumoral and hormonal local regulatory mechanisms were suspected because adrenergic agonists and antagonists as well as some glycoprotein hormones were shown to lower IOP. Pharmacologic doses of epinephrine and other agents were shown to cause changes in outflow facility which were smaller than the variations of IOP seen in the circadian pattern [Sears et al., 1981; Caprioli and Sears, 1984], and both adrenergic agonists and antagonists were known to cause changes in AHF. It therefore appeared

that AHF was the component of aqueous humor dynamics most affected by the hypothetical regulatory mechanism and most likely to be responsible for the circadian rhythm of IOP, consistent with the earlier conclusion of Katavisto [1964] that AHF plays the most important role in diurnal variations of IOP.

In order to identify a possible mechanism of AHF and IOP regulation a system devoid of all the complexities encountered with *in vivo* studies was needed. Animal and human ciliary process tissue provided one such *in vitro* system in which the effects of adrenergic drugs and other agents could be determined. Investigations utilizing enzyme stimulation, pharmacologic binding, and fluorescent analogue localization techniques resulted in the discovery of adenyl cyclase-coupled beta-adrenergic receptors in ciliary process tissue. Correlations between *in vitro* AC stimulation and *in vivo* AHF diminution caused by adrenergic agonists and other compounds led to a theory of regulation of IOP by the ciliary epithelial adenyl cyclase receptor complex.

CyclicAMP phosphodiesterase was found in rabbit ciliary process tissue in 1966 [Shanta et al, 1966]. Shortly thereafter AC was identified in the same tissue, and its activation by catecholamines, KCl, NaF, and PGE1 was established [Waitzman & Woods, 1971]. The order of AC stimulatory potency of the catecholamines (isoproterenol = epinephrine > norepinephrine = phenylephrine) was characteristic of a beta-adrenergic pattern [Waitzman and Woods, 1971] and suggested that the AC present in ciliary processes might be associated with beta-receptors in a fashion similar to the AC-catecholamine receptor coupling known to

occur in other tissues [Robison et al., 1967].

Pharmacologic binding studies demonstrated the presence of adrenergic receptors in the membranes of homogenized rabbit iris-ciliary body [Neufeld and Page, 1977]. With a fluorescent propranolol analogue, beta-receptors were localized to ciliary epithelial membranes, ciliary processes, and episcleral limbal blood vessel walls in the rabbit eye [Dafna et al., 1979]. At the same time, rabbit ciliary process AC was localized ultrastructurally (by a cytochemical electron microscopy localization technique) to the nonpigmented epithelial (NPE) cell plasma membranes, NPE-pigmented epithelial cell boundary, and ciliary process stromal capillary endothelial cells [Tsukahara and Maezawa, 1978]. These data suggested the presence of beta-receptors and adenyl cyclase together in the ciliary process (possibly NPE) epithelial membranes, sites which were known to be involved in the secretion of aqueous humor [Sears, 1980].

In 1980 Bromberg et al. demonstrated that beta-receptors and AC existed together in the same particulate fraction of discontinuous sucrose gradients of homogenized rabbit ciliary processes. The receptors were identified by ^{125}I-hydroxybenzylpindolol (^{125}I-HYP) binding and characterized as beta-receptors by the ability of antagonists (timolol, l-alprenolol, and d,l-propranolol) and agonists (l-norepinephrine, l-isoproterenol, and phentolamine) to displace the bound ^{125}I-HYP. Binding to the receptors was greater for timolol and other beta-antagonists than for beta-agonists (isoproterenol > norepinephrine), similar to the binding affinities documented with beta-receptors of

other tissues. AC was stimulated by NaF. In the same year Nathanson identified AC activity in rabbit, cat, dog, monkey, and human ciliary processes. In the rabbit, AC was stimulated by adrenergic agonists with a potency order: isoproterenol > epi > norepi > phenylephrine, and isoproterenol stimulation of AC was inhibited by propranolol and timolol. The data from Nathanson's and another study [Cepelik and Cernhorsky, 1981] indicated that rabbit AC was stimulated by a beta $_2$-receptor. Preliminary studies suggested that human ciliary process AC also exhibited a beta $_2$-adrenergic receptor stimulatory pattern; this suggestion was confirmed by a subsequent analysis [Nathanson, 1981].

Gregory et al. [1981a] demonstrated in rabbits that cholera toxin lowered IOP, decreased AHF, and increased anterior uveal blood flow when administered in dosages and by routes expected for endogenous mediators. Cholera toxin also stimulated rabbit ciliary process AC [Gregory et al., 1981b; Sears et al., 1981] and bound to the interdigitating and apical plasma membranes of the ciliary process nonpigmented epithelium [Mishima et al., 1982]. Subsequently it was demonstrated that forskolin, a direct stimulator of AC, lowered AHF and IOP [Caprioli and Sears, 1983; Caprioli et al., 1984] while potently stimulating AC [Caprioli and Sears, 1984]. These results indicated that several agents lowered AHF and IOP as well as increased cAMP via receptor-mediated or direct stimulation. Although no ciliary process receptors specific for glycoprotein hormones were documented it was shown that hCG, FSH, and other glycoprotein hormones, known to stimulate AC by activating AC-coupled receptors, lowered IOP [Sears and Mead, 1983].

Based upon the knowledge that stimulation of AC of the NPE plasma membranes resulted in decreased AHF and IOP, that cholera toxin acted on other epithelia to cause movement of fluid from the basal to the apical portion of the cell, and that cholera toxin, forskolin, and isoproterenol caused enlargement of the ciliary channels between the NPE and PE apices [Caprioli and Sears, 1984], two investigators postulated the manner in which AC stimulation would lead to a decrease in AHF [Caprioli and Sears, 1984; Sears, 1985]. The orientation of other epithelia on which cholera toxin is known to act is with the apical portion of the cell adjacent to a lumen (into which fluid is secreted when cholera toxin activates AC and increases cAMP). In the eye, because of invagination of the optic vesicle during development [Jakobiec, 1982], the orientation of the apical portion of the NPE cell is not adjacent to the lumen (i.e. the posterior chamber) but instead is adjacent to the pigmented epithelial cell. If cholera toxin (and other AC stimulators) cause movement of fluid in all epithelial cells from the base to the apex and thence across the apical membrane, then its effect in stimulating the AC of NPE cells would be movement of fluid across the apical membrane into the ciliary channel between the NPE and PE cells. Stimulation of AC and movement of fluid would result in the enlargement of the ciliary channels, a phenomenon observed when AC stimulation was effected by cholera toxin, forskolin, and isoproterenol [Fujita et al., 1984]. From the ciliary channels fluid could move to the ciliary stroma and be reabsorbed by the stromal capillaries. The movement of fluid away from the posterior chamber across the NPE cell into the ciliary channels, with or without subsequent movement into the ciliary stroma, would bring about a net decrease of aqueous humor inflow. Thus the

authors [Caprioli and Sears, 1984; Sears, 1985] proposed the theory for the adenyl cyclase-receptor complex located in the ciliary NPE cell which would regulate aqueous humor formation and IOP. Substances able to stimulate the AC either via the receptor (as in the case of beta-adrenergic agonists, glycoprotein hormones, and cholera toxin) or directly (as in the case of forskolin) would bring about a diminished AHF and IOP.

The discovery of the adenyl cyclase-receptor complex of the ciliary NPE cell and the correlation of AC stimulation with lowering of AHF and IOP in rabbits and man led to an exciting hypothesis for a regulatory mechanism controlling IOP via AHF. This hypothesis provides an explanation for the mode of action of beta-agonists, glycoprotein hormones, forskolin, and cholera toxin in lowering AHF, but what about timolol and other beta-blockers which also lower AHF? Although timolol does lower IOP it has no effect on the adenyl cyclase or cAMP phosphodiesterase activity of the rabbit ciliary process tissue [Gregory et al., 1981b] and therefore must act via some other mechanism than this AHF-lowering AC-receptor complex. The mode of action of timolol and other beta-blockers remains to be discovered.

G. The Adenyl Cyclase-Receptor Complex and Phosphodiesterase Inhibitors

Knowing that rabbit ciliary processes contain cAMP phosphodiesterase [Shanta et al, 1966; Sears, 1978] as well as AC and that stimula-

tion of AC results in lowering of IOP, the question of the effects of a phosphodiesterase inhibitor on this adenyl cyclase receptor complex and IOP has been raised. In tissues in which AC stimulation and cAMP formation has been established to mediate a response to drugs or hormones, investigators have attempted to confirm the role of cAMP by demonstrating potentiation of the agent's effect by phosphodiesterase inhibitors [Sutherland et al., 1968; Robison et al, 1971; Poch and Reich, 1983] or by replication of the agent's effect by a phosphodiesterase inhibitor alone when the basal turnover rate of cAMP is high [Wells and Kramer, 1981]. Phosphodiesterase inhibitors should potentiate or rep- licate an AC stimulator's effect by preventing the degradation of cAMP by its phosphodiesterase [Robison et al, 1971]. In the eye, as in other tissues, it is hypothesized that a phosphodiesterase inhibitor would replicate the effect of AC stimulators if basal production of cAMP in the ciliary NPE was high or at least would potentiate the effect of AC stimulators in the event that basal production of cAMP might be too low for a phosphodiesterase inhibitor alone to cause a significant increase of cAMP. A phosphodiesterase inhibitor alone or in combination with an AC stimulator would be expected therefore to cause an increase in cAMP in ciliary NPE cells and a resultant decrease in AHF and IOP.

HL725 (Hoechst-Roussel Pharmaceuticals, Inc.) has been shown to be a potent inhibitor of cAMP phosphodiesterase [Ruppert and Weithmann, 1982] and is believed to inhibit platelet aggregation and to cause peripheral vasodilation via its cAMP phosphodiesterase inhibitory action [Lal et al., 1981; Ruppert and Weithman, 1982; Lal et al., 1984]. Isoproterenol is known to stimulate rabbit ciliary process AC [Gregory et

al., 1981a; Schmitt et al., 1981] and to increase the size of ciliary channels [Fujita et al., 1984] and is believed to decrease IOP primarily by decreasing AHF [Weekers, 1955; Eakins, 1963; Bonomi, 1964b; Ross and Drance, 1970; Langham et al., 1971; Gaasterland et al., 1973; Takase, 1976; Mishima, 1982]. It was of interest therefore to investigate the effects of HL725, alone or in conjunction with low doses of isoproterenol, on the IOP of rabbits and on the cyclic nucleotide production of isolated rabbit ciliary processes.

II. MATERIALS AND METHODS

A. Rabbit IOP Experiments:

1. Rabbit Housing Facility Environment: New Zealand male albino rabbits weighing 2-2.5 kg. were kept in the animal care housing facility in individual cages with food and water available for ad lib intake. Room temperature was 22 °C. The rooms in which the animals were housed were maintained on a 12:12 hour light:dark schedule with wide spectrum fluorescent lighting in use between 6:00 a.m. and 6:00 p.m. controlled by an automatic timing device. Indirect light from the hallway's fluorescent lights entered the rooms through glass-windowed doors between 6:00 and 7:00 p.m. Between 7:00 p.m. and 6:00 a.m. no artificial overhead lights were in use in the rooms or the hallway, but because the rooms were not "light tight" they were exposed to some extraneous light (from small emergency lights in the hallway) during the periods of darkness of the light:dark schedule.

Rabbits were kept in the housing facility for a minimum of one day and a maximum of three days before the commencement of experimental procedures.

2. IOP Measurement Procedure: Rabbits were placed individually upon

a table in the housing facility room and were allowed to become quiet and relaxed before IOP measurements were obtained. Each eye was examined to determine pupil size and degree of conjunctival and perilimbal injection. One drop (50 microl.) of proparacaine hydrochloride (Ophthetic) 0.5% solution was instilled in each eye, and the eyelids were gently retracted without applying pressure to the globe. After the rabbit's extraocular muscles had relaxed, eyelid retraction was released and IOP was measured with a Digilab 30-D applanation pneumatonometer sensor membrane placed tangentially on the cornea until a consistent reading of 5 seconds duration was obtained.

When IOP measurements were taken during the dark period of the light:dark schedule a small flashlight was used to locate the animal in its cage and to provide indirect light during the measurement period.

3. Pneumatonometer Calibration Procedure: The Digilab 30-D applanation pneumatonometer was calibrated for rabbit eyes by the following method. Rabbits were sacrificed with 3-5 cc. of sodium pentobarbital (Nembutal) administered intravenously. One set of calibrations was obtained on eyes remaining in their orbits, and another set was obtained on enucleated eyes (stabilized on saline-soaked gauze in the upper portion of a small beaker). Throughout the calibration procedure the eyes were kept moist with periodic topical applications of saline. The anterior chamber of each eye was cannulated by injecting a 20-gauge needle through the sclera (approximately 1 mm. from the corneoscleral limbus) using a needle gun. The needle was connected via polyethylene

tubing and a three-way stopcock to a saline reservoir. The reservoir heights were labeled with 5 mm.Hg. graduations from zero to 50 mm.Hg. (using the conversion of 13.5 mm.Hg./mm. saline). Beginning at 10 mm.Hg. and progressing to 50 mm.Hg., the height of the reservoir was increased by increments equivalent to 5 mm.Hg. After each adjustment of the reservoir level to a new height the pressure in the eye was allowed to reach an equilibrium for five minutes. At each level of manometric pressure three tonometric pressure readings were recorded both with the stopcock open to the saline reservoir throughout the measurement procedure and with the stopcock having been closed to the saline column immediately before each individual tonometric measurement. Measurements taken with the Digilab 30-D applanation pneumatonometer were recorded when a consistent reading of 5 seconds duration was obtained.

4. Documentation of Circadian Rhythm of IOP: A consistent pattern of IOP was noted in almost all rabbits over the course of several months of experimental procedures, even when the rabbits used had been in the housing facility as little as one day or had been exposed to light from a flashlight during the darkness period of the light-dark schedule. It was decided therefore to document the circadian rhythm present under those conditions. Four rabbits which had been in the housing facility for 2 days were utilized to determine the circadian rhythm. During the 2 days entrainment period the rabbits were exposed to indirect light from a flashlight utilized during the darkness period to locate other rabbits and measure IOP in the room. During the following 24 hour period IOP measurements were taken under the regular fluorescent lighting

during the 6:00 a.m. to 6:00 p.m. light period and with indirect light (from a flashlight placed approximately 12 inches away from the rabbit with the beam angled at 90 degrees from the rabbit) during the 6:00 p.m. to 6:00 a.m. dark period.

The IOP circadian rhythm of these four rabbits was compared to the IOP circadian rhythm of control eyes from HL725 intravitreal injection studies. Rabbits which were kept in another housing facility without a consistent light-dark cycle or on whom measurements were taken at significantly different times of the day were excluded from the control eyes circadian study group.

5. Preparation of Solutions and Suspensions of HL725: All solutions and suspensions were prepared and stored at room temperature in polystyrene test tubes.

HL725 was supplied by Hoescht-Roussel Pharmaceutical, Inc.

a. Solutions and Suspensions in Saline: HL725 was soluble at concentrations between 0.05 and 0.2%. Suspensions were placed in an ultrasonic bath to reduce particle size and were resuspended by swirling immediately before topical applications or intravitreal injections were made. Fresh solutions and suspensions were prepared every two to three weeks although stability at room temperature for as long as three months was documented by systemic hypotensive effectiveness when administered intravenously to rabbits whose blood pressure was monitored by

femoral artery catheterization. Sterile saline, syringes, and test tubes were used in preparing solutions and suspensions, and fresh solutions were prepared the day prior to intravitreal injections.

HL725 0.1% and 1% suspensions had pHs ranging from 4.75 to 4.35 and 3.6 to 3.45, respectively. pH-adjusted saline was used in control eyes.

b. Suspensions in BSS and Hydroxypropylmethylcellulose: HL725 was less soluble in BSS than in saline and at concentrations greater than $4\text{-}5 \times 10^{-5}$ M formed a white flocculent precipitate. Topical suspensions of 1%, 2.5%, and 5% HL725 were prepared in Balanced Salt Solution (BSS) with 0.5% hydroxypropylmethylcellulose (Isoptalkaline) and had pHs of 6.0-6.5, 4.5-5.0, and 3.0-3.5, respectively. A solution of 0.5% hydroxypropylmethylcellulose in BSS (pH 7.0) was used as a control.

6. Other Solutions:

a. Isoproterenol: Isoproterenol (Isuprel) 1% in buffered aqueous solution with preservatives was stored in its light-proof container at room temperature. Dilutions in saline were prepared within 30 minutes preceding each experiment, and exposure to light was minimized until topical applications were made.

b. Ophthetic: Propracaine hydrochloride (Ophthetic) 0.5% solution,

pH 4.5-5.0, was utilized to obtain topical anesthesia prior to applanation tonometry and prior to intravitreal injections.

7. Topical Applications: 50 microl. of HL725 solutions and suspensions were instilled in right eyes. 50 microl. of control solutions were instilled in left eyes. Isoproterenol solutions and their saline controls were instilled in volumes of 25 microl. in right and left eyes, respectively.

8. Intravitreal Injections: Intravitreal injections of 10 microl. were delivered with a 50 microl. Hamilton syringe through a 30-gauge needle into the anesthetized, proptosed rabbit eye. HL725 solutions freshly prepared in sterile saline were delivered into right eyes, and sterile saline was delivered into left eyes. HL725 solutions and suspensions were prepared in concentrations appropriate to result in final intravitreal concentrations ranging between 10^{-6} and 10^{-10} M.

B. Rabbit Ciliary Process Cyclic Nucleotide Stimulation Experiments:

1. Tissue Dissection Procedure: Male albino New Zealand rabbits weighing 2-2.5 kg. were sacrificed individually (immediately prior to eye dissection procedures) by intravenous injection of 3-5 cc. sodium pentobarbital (Nembutal). Blood was drained from the head by holding

the rabbit upright by the back of the neck for approximately ten seconds. Eyes were enucleated and placed in a beaker of cold saline (0-4 °C) chilled on ice. Subsequent dissection procedures were performed individually on each eye.

The eye was placed on BSS-soaked tissues on a metal bar (chilled with an ice-filled port) while extraneous conjunctival and extraocular muscle tissue was trimmed from the eye. The posterior pole of the eye was incised, and five incisions (through the scleral, choroidal, and retinal layers) were extended radially from the posterior pole to a point approximately 1-2 mm. anterior to the equator. The lens, zonule, and most of the vitreous body were freed from the anterior segment using a lens loop. Retinal and choroidal tissue were removed from the sclera with gentle strokes with a cotton-tipped swab. The preparation was transferred and secured to a Plexiglas holder within a small Petri dish. The Petri dish was filled with cold BSS (0-4 °C) and placed on ice. Using a dissecting microscope and dissection instruments, individual ciliary processes were cut away from the ciliary body and iris. Ciliary processes were placed in individual 1 ml. PotterElvehjem tissue homogenizers full of BSS chilled on ice.

The entire procedure was repeated until ciliary processes were harvested from a total of four eyes.

2. Ciliary Process Incubation Procedure: BSS was removed from each homogenizer to give a remaining volume of 300 microl. HL725 reagent

solutions and suspensions in BSS (prepared the morning of the procedure) were warmed to 30°C in a water bath. Tissue homogenizers (containing ciliary processes in 300 microl. BSS) were placed in the 30°C water bath 5 minutes before individual reagent solutions were to be added. After the addition of 100 microl. of reagent solution, the ciliary processes and reagent solution were incubated for ten minutes. At ten minutes 30 microl. of TCA solution containing tritiated cAMP were added, and the ciliary processes were homogenized with 30-40 strokes of a motor-driven Teflon pestle in the glass homogenizers. The preparation then was pelleted by centrifugation of the homogenized solutions at 1900-2100 x g. in an International Clinical Centrifuge. The supernatants were transferred into labeled test tubes and frozen at -20°C. 500 microl. of 1 N. NaOH solution were added to each pellet, and the mixtures were allowed to stand at room temperature for a minimum of three days.

NOTE: Tissue dissection and incubation procedures later were done with saline in place of BSS because of HL725's lesser solubility in BSS than in saline.

3. TCA Extraction Procedure, Preparation of Dilutions for RIA, and Determination of V_T:

a. Supernatants (from procedure 2 above) were allowed to reach room temperature. One Pasteur pipetteful of water-saturated ether was added to each tube, and after vortexing the mixture most of the ether

layer was removed by pipetting. The ether extraction of TCA was repeated five times. The tubes were then warmed in a 55 °C water bath for two hours. After the tubes were allowed to cool, the remaining solutions were used to prepare dilutions for the RIA and to determine the V_T of each sample.

b. Appropriate dilutions for the RIAs were prepared with acetate buffer solution and were then frozen at -20 °C until the RIAs were performed.

c. The V_T of each tube was determined by comparing radioactivity disintegrations per minute (determined in a 1219 Rackbeta Liquid Scintillation Counter) for 100 microl. volume of samples (dissolved in 1 1/3 ml. of Ultrafluor) with d.p.m.s for 30 microl. of tritiated cAMP - TCA solution (combined with 70 microl. of distilled water and dissolved in 1 1/3 ml. of Ultrafluor). The relationship:

$$\text{Total} = (\text{conc.}) \times (V_T)$$

or:

$$V_T = \{(\text{d.p.m. for tritiated cAMP-TCA soln.}/\text{d.p.m. for sample}) \times 100\}$$

was used to calculate each sample's V_T.

d. Any solutions remaining (after the dilutions and V_T determinations had been made) were frozen at -20 °C.

4. Protein Concentration Determination by Lowry Assay: This assay was performed in triplicate for each sample and standard solution. Standard protein solutions (0-60 microg. BSA/100 microl. 1 N. NaOH) were prepared from stock BSA solution (1.5 mg. BSA/ ml. 1 N. NaOH). The sample pellets dissolved in 1 N NaOH, which had been allowed to stand (in tissue homogenizers) at room temperature for a minimum of three days, were vortexed. 100 microl. of each standard and sample solution were pipetted into individual borosilicate test tubes. 1 ml. of a 1:1:100 1% $CuSO_4$: 2% KNatartrate: 2% Na_2CO_3 solution was added to each tube. After mixing by vortex each sample was allowed to stand 15-20 minutes. 100 microl. of 1 N. Phenol Reagent (Folin-Ciocalteau) solution were added to each tube while vortexing. After one hour had elapsed, 300 microl. of each sample were pipetted into individual wells of a titertek plate, with the first row of wells of the plate filled with a mixture of the three zero-standard solutions.

Absorbance at 640 nm. was read by a Titertek Multiskan. A standard curve of absorbance vs. concentration was plotted, and each sample's protein concentration was determined.

5. Determination of Cyclic Nucleotide Concentration by cAMP and cGMP RIAs: Biomedical Technologies Inc. cyclic nucleotide RIA kits were utilized. Duplicates of standards and samples were used. The previously prepared and frozen sample dilutions as well as the RIA kit solutions were allowed to warm to room temperature. Cyclic nucleotide standard solutions (5-500 fM/100 microl.) were prepared. Both the standard

and sample solutions were acetylated with 5 microl. of freshly prepared acetic anhydride:triethylamine 1:2 mixture. 100 microl. of tracer solution were added to each tube. 100 microl. of antiserum were added to all but the first two tubes, to which nonspecific binding reagent was added. The racks of tubes were swirled gently for 60 seconds, covered with foil, and incubated at 4 °C for 18-20 hours.

After 18-20 hours incubation all tubes then were placed on ice, and 1 ml. acetate buffer was added to each tube. The tubes were centrifuged at 5000 r.p.m. for 15 minutes at 0-4 °C in a Sorvall RC5B Refrigerated Superspeed Centrifuge. The supernatants were removed by pipetting. Each pellet was counted for one minute in a Searle Analytic Inc. Model 1190 Automatic Gamma System.

The standard curve of percent binding vs. cyclic nucleotide concentration was plotted, and the cyclic nucleotide concentration of each sample was determined from its percent binding. Final cyclic nucleotide concentration (in pM. cyclic nucleotide/ mg. protein) was calculated by taking into account each sample's acetate buffer dilution, V_T, and protein concentration, as well as the tritiated cAMP added.

C. Data Analysis: Linear regression analyses, statistical calculations, and graphing of data were done with the Clinfo Systems Computer Programs.

III. RESULTS:

A. Rabbit IOP Experiments:

1. Pneumatonometer Calibration for Rabbit Eyes:

All calibration data exhibited linear relationships between pneumatonometer IOPs and manometer IOPs. Linear regression analysis was performed on tonometer versus manometer IOP for each rabbit to determine the slope and standard error of estimate of each line. Mean slope ± SEM was calculated for each calibration condition (eyes in orbits: open or closed stopcock; enucleated eyes: open or closed stopcock). The mean slopes of the pneumatonometer calibration curves for rabbit eyes in their orbits were (mean ± SEM, n=5) 0.83 ± 0.01 and 0.98 ± 0.02 under conditions of open and closed stopcocks, respectively. The mean slopes of the calibration curves for enucleated rabbit eyes were 0.82 ± 0.02 and 1.04 ± 0.02 with open and closed stopcocks, respectively. All curves had y-intercepts equal to 0.

To simplify the representation of pneumatonometer calibration curves, they were drawn by linear regression analysis of mean tonometer IOPs and manometer IOPs for pooled data for each calibration condition. (See figures 1-4.) These curves had slopes (eyes in orbits: open

stopcock: 0.83 $\pm$ 0.01 and closed stopcock: 0.98 $\pm$ 0.03; enucleated eyes: open stopcock: 0.82 $\pm$ 0.03 and closed stopcock: 1.04 $\pm$ 0.03) similar to the mean slopes and SEMs calculated from calibration curves of individual eyes.

Pooled variances and the t-statistic for comparison of two slopes were calculated to determine the statistical significance of the differences between slopes of the calibration curves obtained under different conditions. The difference between mean slopes of lines obtained with open stopcocks in comparison to those obtained with closed stopcocks was significant at $p< 0.001$ for both enucleated eyes and eyes in their orbits. In contrast, the difference between mean slopes of lines obtained with enucleated eyes in comparison to those obtained with eyes in their orbits was statistically insignificant under both open and closed stopcock conditions. The t-statistic for comparison of mean slopes to the constant 1.0 revealed that the mean slopes of lines obtained with open stopcocks were significantly ($p<0.01$) different from 1.0 but that the mean slopes of lines obtained with closed stopcocks were not significantly different from 1.0.

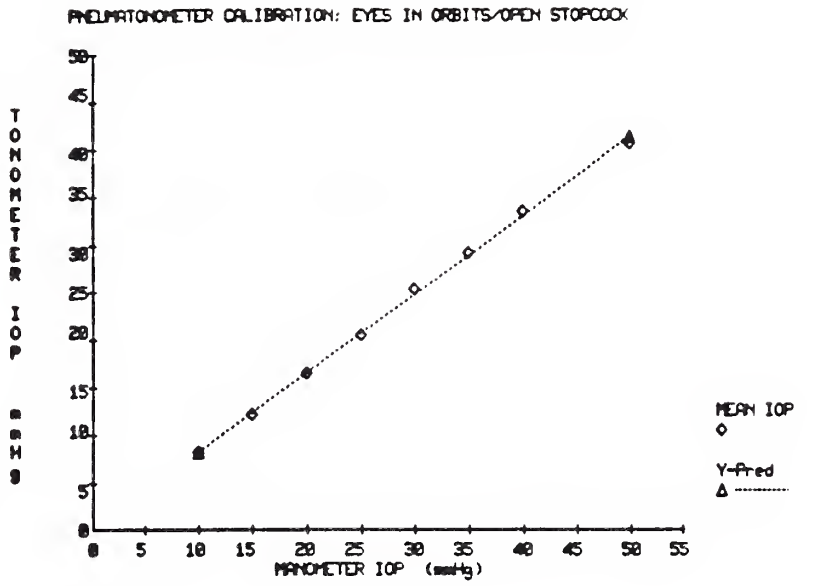


Figure 1: Mean Pneumatonometer IOPs vs. Manometer IOP for rabbit eyes in their orbits (n=5). Calibration data were obtained under open stopcock conditions. Slope = 0.83 ± 0.01.

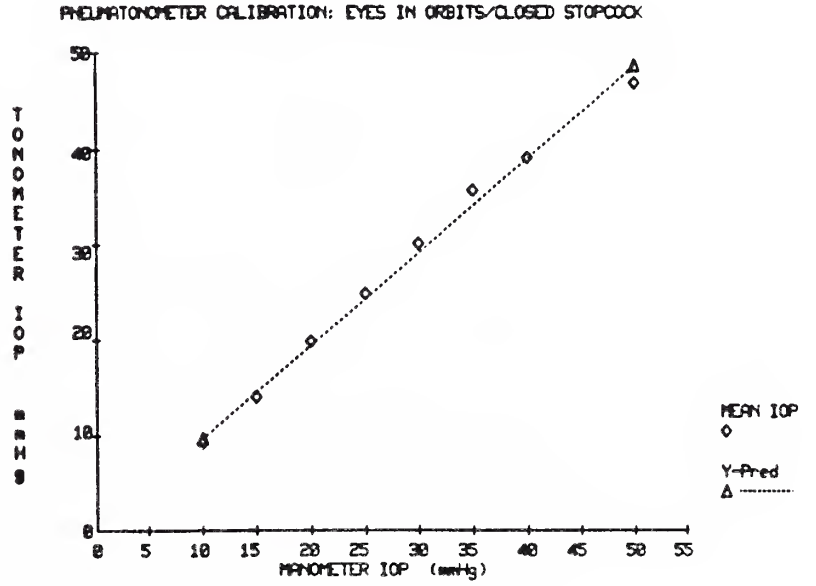


Figure 2: Mean Pneumatonometer IOPs vs. Manometer IOP for rabbit eyes in their orbits (n=5). Calibration data were obtained under closed stopcock conditions. Slope = 0.98 ± 0.03.

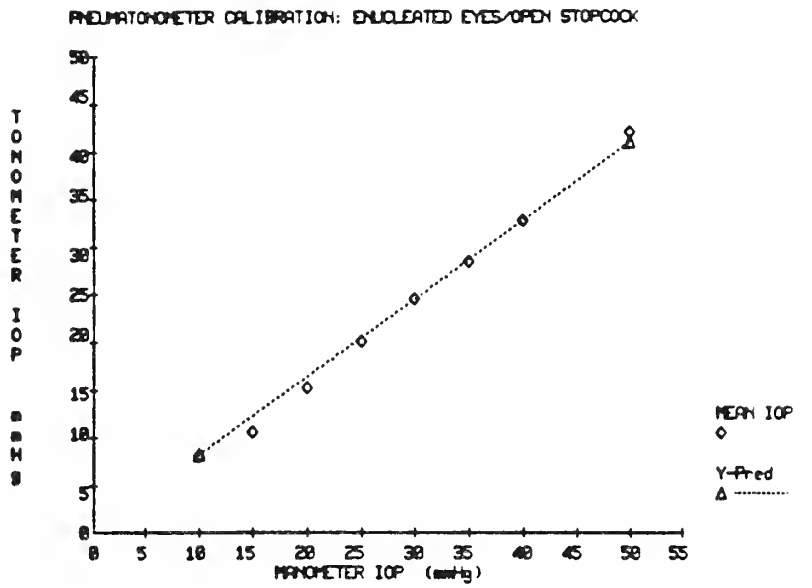


Figure 3: Mean Pneumatonometer IOPs vs. Manometer IOP for enucleated rabbit eyes (n=5). Calibration data were obtained under open stopcock conditions. Slope = 0.82 ± 0.03.

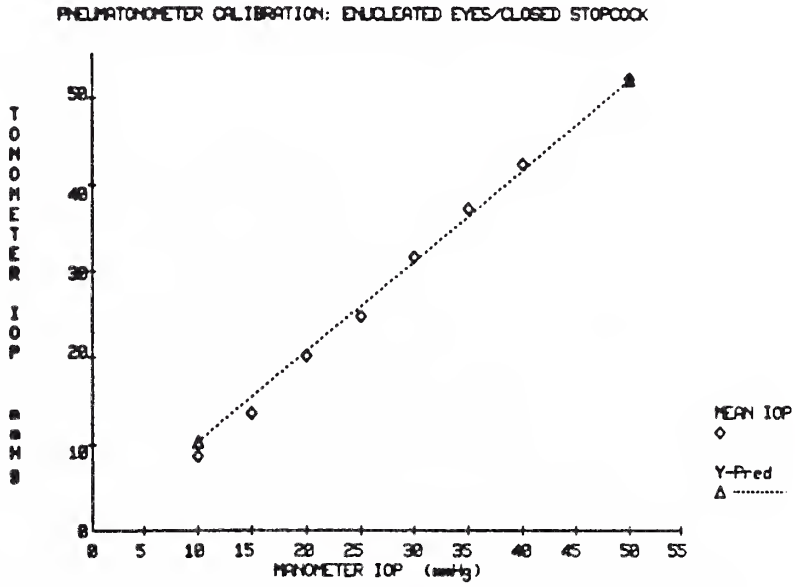


Figure 4: Mean Pneumatonometer IOPs vs. Manometer IOP for enucleated rabbit eyes (n=5). Calibration data were obtained under closed stopcock conditions. Slope = 1.04 ± 0.03.

2. IOP Circadian Rhythm :

{Note: Circadian Time: 0 C.T. = 6:00 a.m.= lights on, and 12 C.T. = 6:00 p.m.= lights out}

See figures 5 and 6 for graphs of the IOP circadian rhythms of circadian study eyes and the HL725 study control eyes. Figure 7 presents both circadian rhythms on the same graph.

The four rabbits which had been in the animal care facility 2 days prior to the commencement of the circadian study demonstrated an IOP circadian rhythm similar to that previously observed in all rabbits (kept in the same environment) which had been used for the HL725 studies. IOP fell during the morning and rose in the afternoon and early evening. At one hour circadian time the first IOP reading was taken, and at 25 hours (or one hour circadian time the next day) the final IOP measurement was obtained. IOP at one hour circadian time (CT) on day 1 and day 2 was 20.5 $\pm$ 0.4 mm.Hg. and 19.6 $\pm$ 0.3 mm.Hg., respectively. Between one and two hours CT the IOP rapidly fell to 16.3 $\pm$ 0.4 mm.Hg. and remained near 16 mm.Hg. until five hours CT. Between five and six hours CT, IOP began a rapid rise to a small peak, 20.9 $\pm$ 0.5 mm.Hg., at nine hours CT and then to the higher recorded peak, 23.6 $\pm$ 0.4 mm.Hg., at thirteen hours CT. Two IOP measurements were taken during the dark period of the light-dark cycle, at 13 and 17 hours CT.

In this study the minimum IOP, 16.3 $\pm$ 0.4 mm.Hg., occurred at two hours CT, and the maximum IOP, 23.6 $\pm$ 0.4 mm.Hg., occurred at 13 hours

CT. The amplitude of the curve between minimum and maximum IOP was 7.4 ± 0.8 mm.Hg.

The circadian rhythm of the control (L) eyes (of HL725 intravitreal injection studies) exhibited an IOP fall in the morning and a rise in the afternoon and early evening. IOP at one hour CT on day 1 and day 2 was 20.2 ± 0.5 mm.Hg. and 18.9 ± 0.5 mm.Hg., respectively. Between one and two hours CT the IOP rapidly fell to 17.3 ± 0.5 mm.Hg and remained near 17 mm.Hg. until five hours CT. Between five and six hours CT, IOP began a rapid rise to a single maximal peak, 22.9 ± 0.4 mm.Hg., at 13 hours CT. Only one IOP measurement was taken during the dark period of the light-dark cycle, at 13 hours CT.

In this group of rabbits the minimum IOP, 17.3 ± 0.5 mm.Hg., occurred at two hours CT, and the maximum IOP, 22.9 ± 0.4 mm.Hg, occurred at 13 hours CT. The amplitude of the curve between minimum and maximum IOP was 6.0 ± 0.5 mm.Hg.

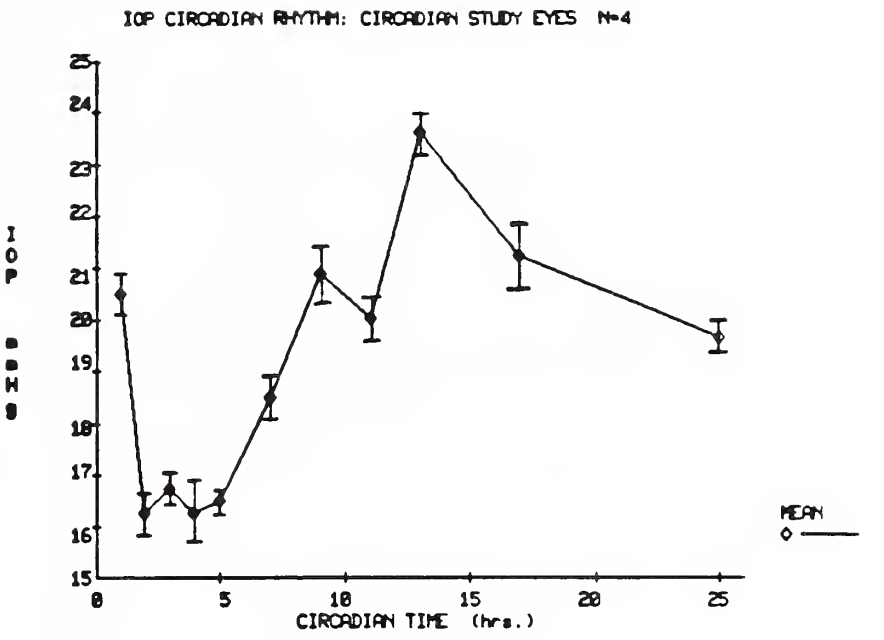


Figure 5: IOP vs. Circadian Time for rabbits after 2 days entrainment to 12 hours light: 12 hours dark. Values represent IOP mean of 8 eyes (n=4) ± SEM.

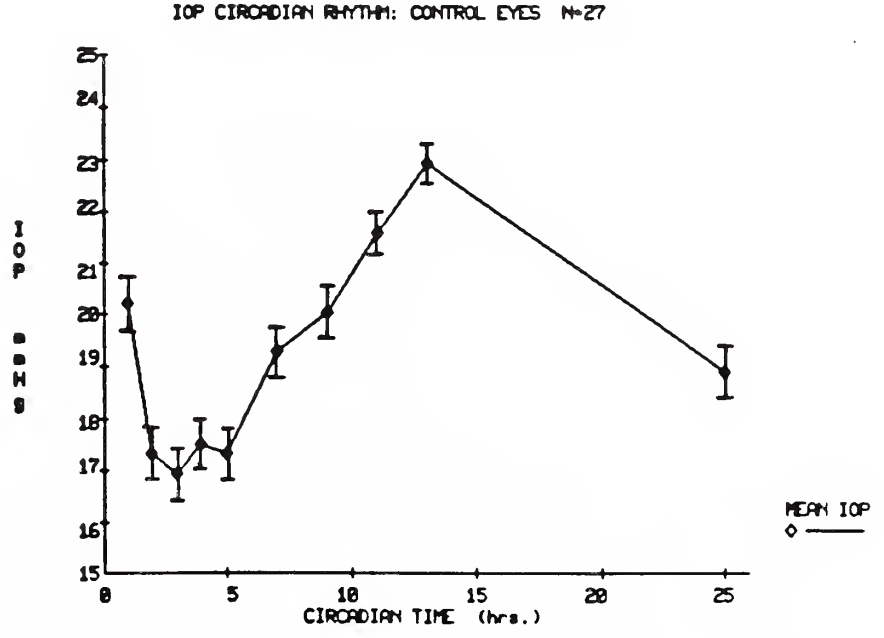


Figure 6: IOP vs. Circadian Time for rabbits after one day to several weeks entrainment to 12 hours light: 12 hours dark. Values represent IOP mean of 27 control eyes (from HL725 intravitreal injection studies) ± SEM.

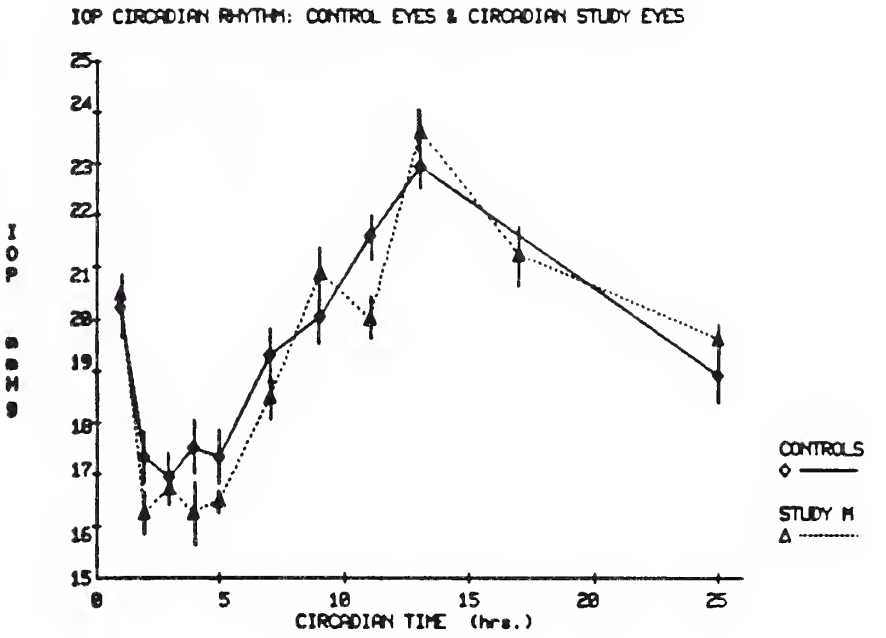


Figure 7: IOP vs. Circadian Time. A comparison of IOP circadian rhythms of circadian study eyes and of HL725 studies control eyes. Values for circadian study eyes represent IOP mean of 8 eyes (n=4) ± SEM. Values for HL725 studies control eyes represent mean IOP ± SEM (n=27).

3. Topical Applications of HL725:

a. 0.1% Solutions and 1% Suspensions in Saline:

Topical application of HL725 0.1% solution (in saline) resulted in a modest, short-term decrease in IOP in the treated eye in comparison to the control eye. The difference in IOP of the treated eye compared to the contralateral control eye (delta (R-L) IOP) at 3 hours was -1.4 mm.Hg. and at four hours was -0.8 mm.Hg. ($p<0.05$, n=8). See figures 8 and 9.

Topical application of HL725 1% suspension (in saline) caused injection of conjunctival and perilimbal vessels of four hours duration with an accompanying bilateral transient hypertension (documented at one hour) and followed by unilateral longer-term hypotension. Delta IOP at 3 hours was -2.3 mm.Hg., at 4 hours -4.0 mm.Hg., and at 8 hours -1.5 mm.Hg. ($p<0.05$, n=4). See figures 10 and 11.

b. 1%-5% Suspensions in BSS/HPMC:

Topical application of HL725 1% suspension in BSS/HPMC resulted in a modest, short-term decrease in IOP with delta IOP at 1 hour -1.4 mm.Hg. and at 2 hours -0.8 mm.Hg. ($p<0.05$, n=8). (See figures 12 and 13.) Topical application of HL725 2.5% suspension in BSS/HPMC caused no significant changes in IOP. (See figure 14.) Topical application of HL725 5% suspension in BSS/HPMC resulted in modest decreases in IOP in the treated eye, with a delta IOP of -1.5 mm.Hg. at 12 hours ($p<0.05$, n=4). (See figures 15 and 16.)

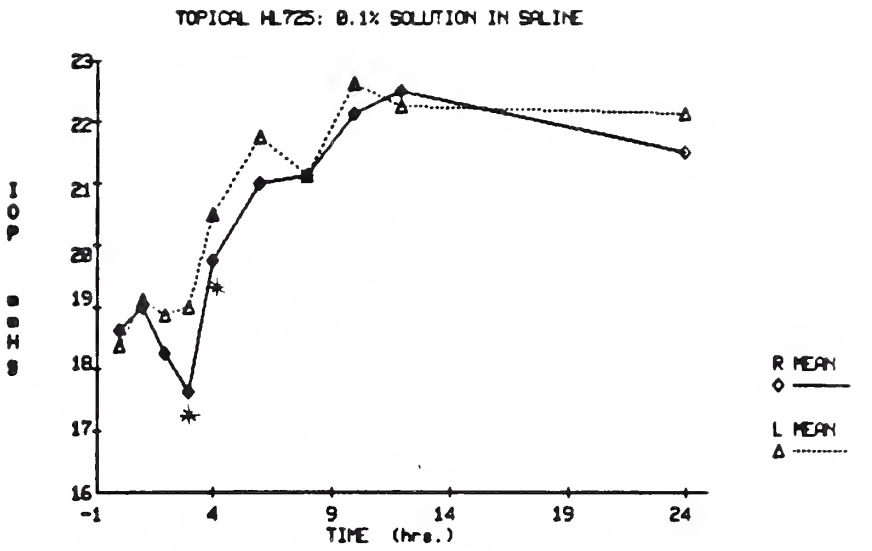


Figure 8: IOP vs. Time for eyes treated with topical applications of HL725 0.1% solution (in saline) OD and of pH-matched saline OS. Values represent IOP means of right and left eyes (n=8). *Significant at $p<0.05$ by paired t-test (two-tailed).

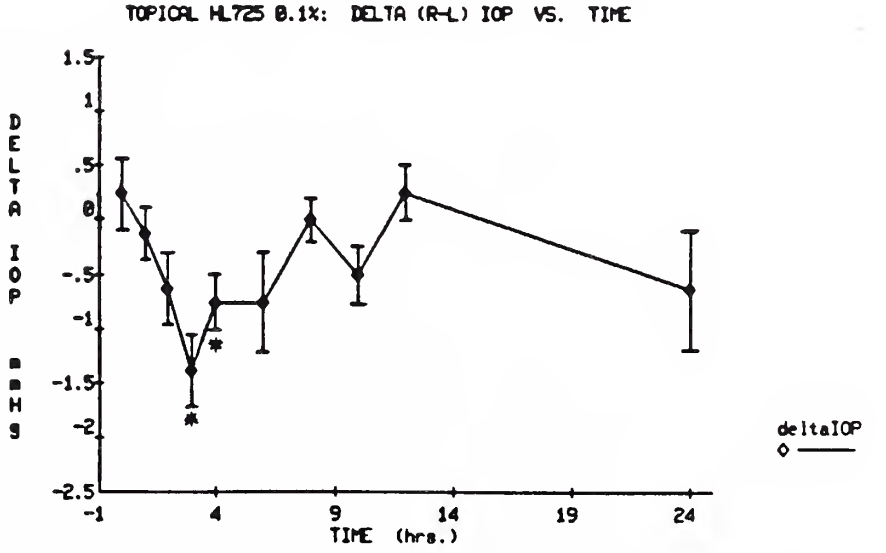


Figure 9: Delta (R-L) IOP vs. Time for eyes treated with topical applications of HL725 0.1% solution (in saline) OD and of pH-matched saline OS. Values represent delta IOP means ±SEMs (n=8). *Significant at $p<0.05$ by two-tailed t-test.

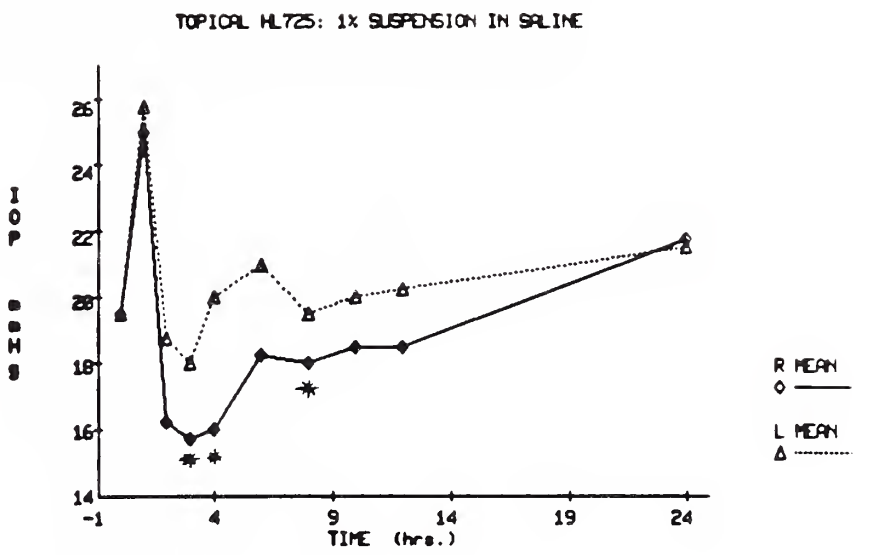


Figure 10: IOP vs. Time for eyes treated with topical applications of HL725 1% suspension (in saline) OD and of pH-matched saline OS. Values represent IOP means of right and left eyes (n=4). *Significant at $p<0.05$ by paired t-test (two-tailed) .

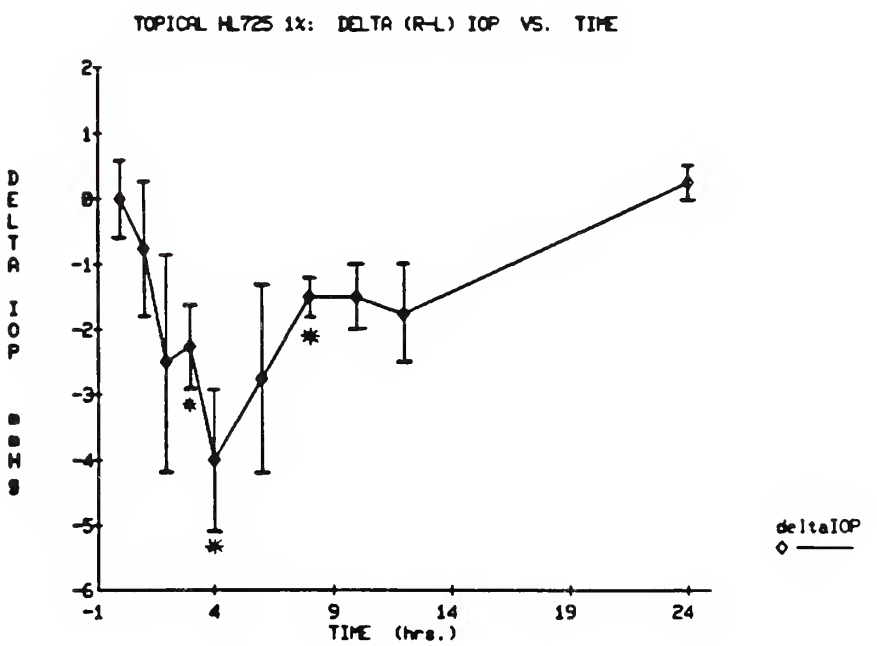


Figure 11: Delta (R-L) IOP vs. Time for eyes treated with topical applications of HL725 1% suspension (in saline) OD and of pH-matched saline OS. Values represent delta IOP means ± SEMs (n=4). *Significant at $p<0.05$ by two-tailed t-test.

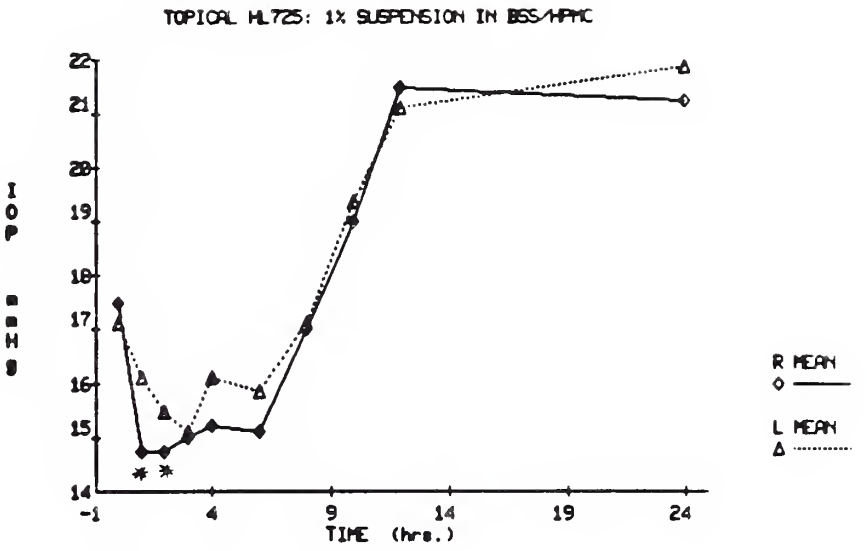


Figure 12: IOP vs. Time for eyes treated with topical applications of HL725 1% suspension (in BSS/HPMC) OD and of saline OS. Values represent IOP means of right and left eyes (n=8). *Significant at $p<0.05$ by paired t-test (two-tailed).

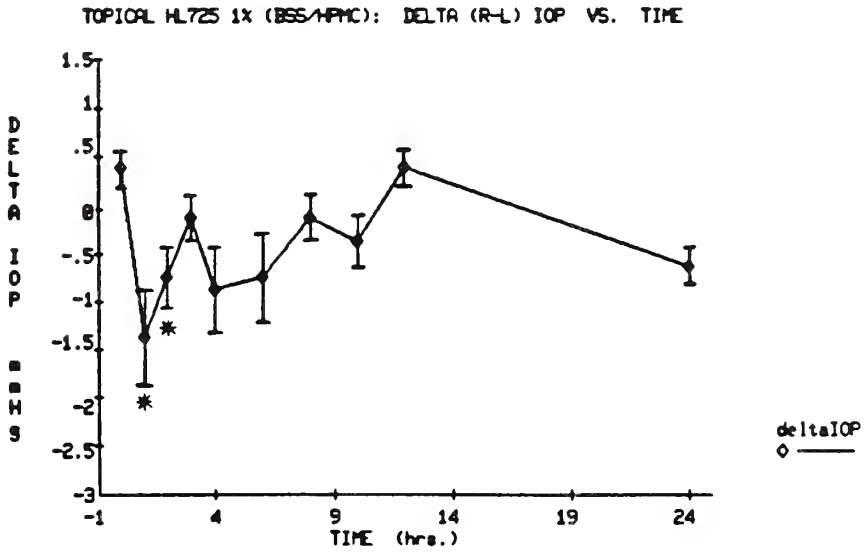


Figure 13: Delta (R-L) IOP vs. Time for eyes treated with topical applications of HL725 1% suspension (in BSS/HPMC) OD and of saline OS. Values represent delta IOP means $\pm$ SEMs (n=8). *Significant at $p<0.05$ by two-tailed t-test.

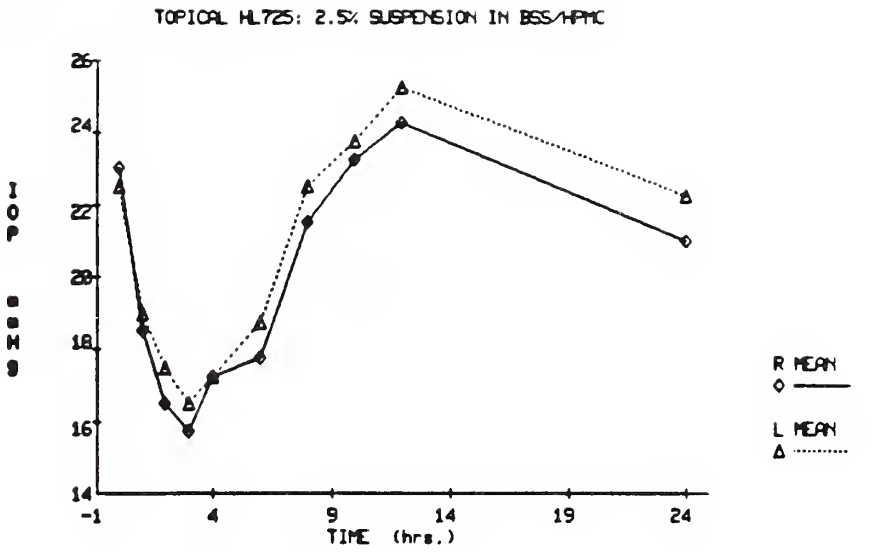


Figure 14: IOP vs Time for eyes treated with topical applications of HL725 2.5% suspension (in BSS/HPMC) OD and of saline OS. Values represent IOP means of right and left eyes (n=4).

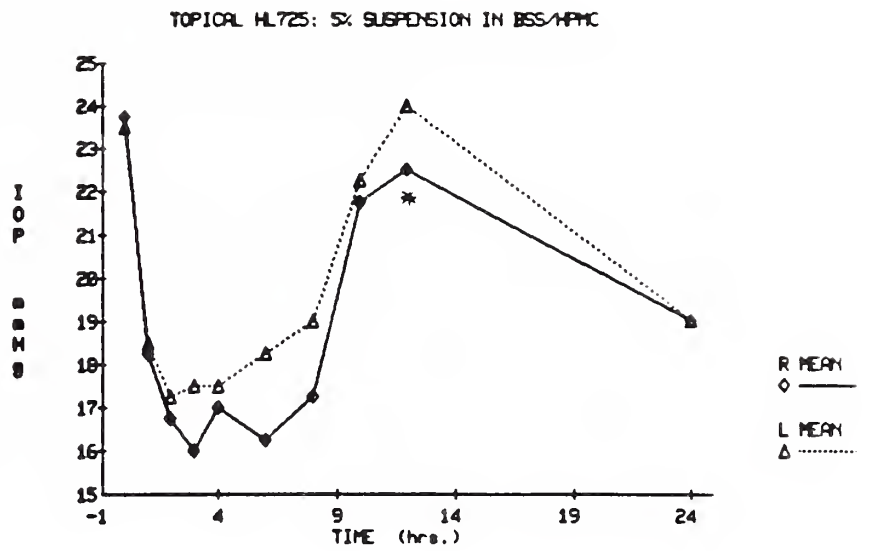


Figure 15: IOP vs. Time for eyes treated with topical applications of HL725 5% suspension (in BSS/HPMC) OD and of saline OS. Values represent IOP means of right and left eyes (n=4). *Significant at $p<0.05$ by paired t-test (two-tailed).

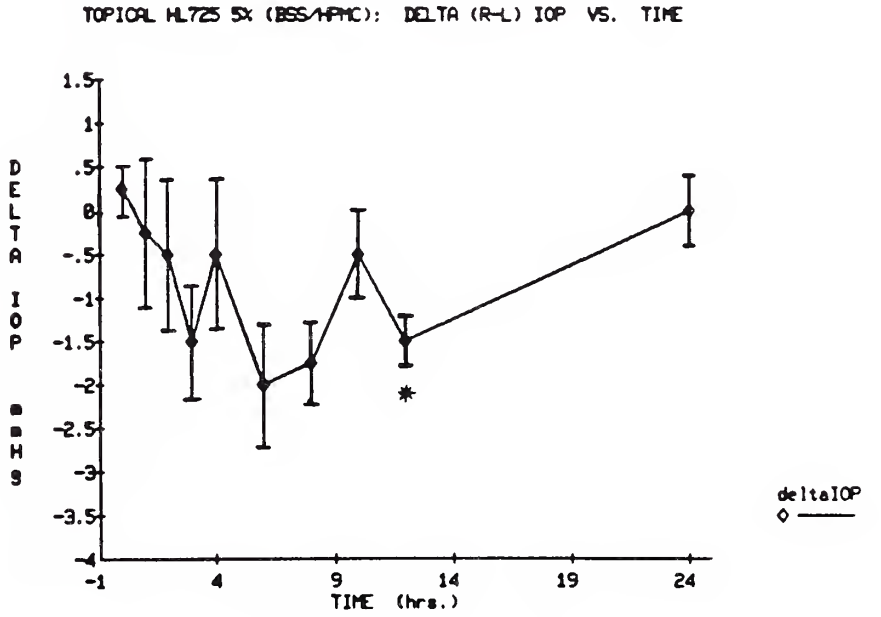


Figure 16: Delta (R-L) IOP vs. Time for eyes treated with topical applications of HL725 5% suspension (in BSS/HPMC) OD and of saline OS. Values represent delta IOP means ± SEMs (n=4). *Significant at $p<0.05$ by two-tailed t-test.

4. Intravitreal Injections of HL725:

Intravitreal injections of HL725 in concentrations between 10^{-6} % and 10% (giving calculated final intravitreal concentrations between 10^{-10} M and 10^{-3} M) effected no significant change in IOP of the study eyes in comparison to the IOP of the contralateral control eyes (treated with intravitreal injection of saline). See figures 17-24.

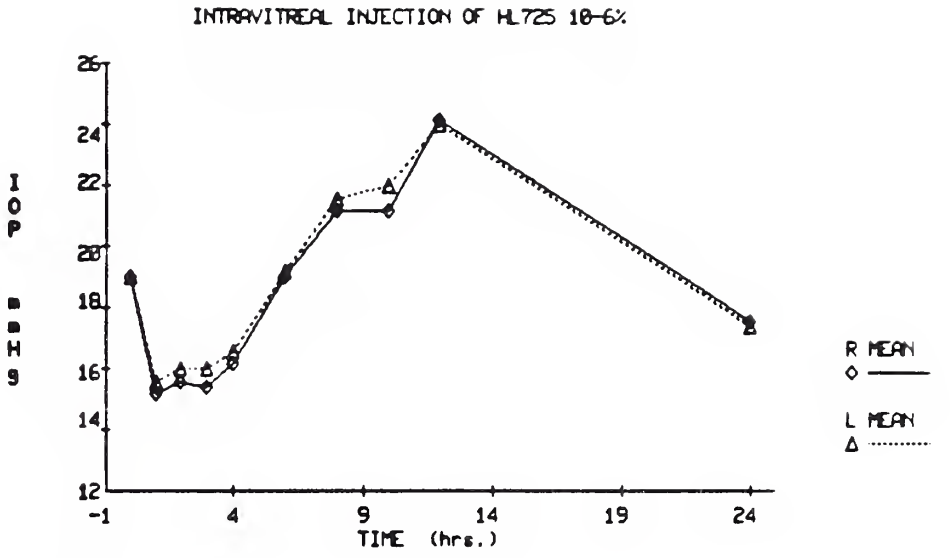


Figure 17: IOP vs. Time for eyes treated with intravitreal injections of HL725 10-6% (final intravitreal concentration of 10-10 M) OD and of saline OS. (n=5)

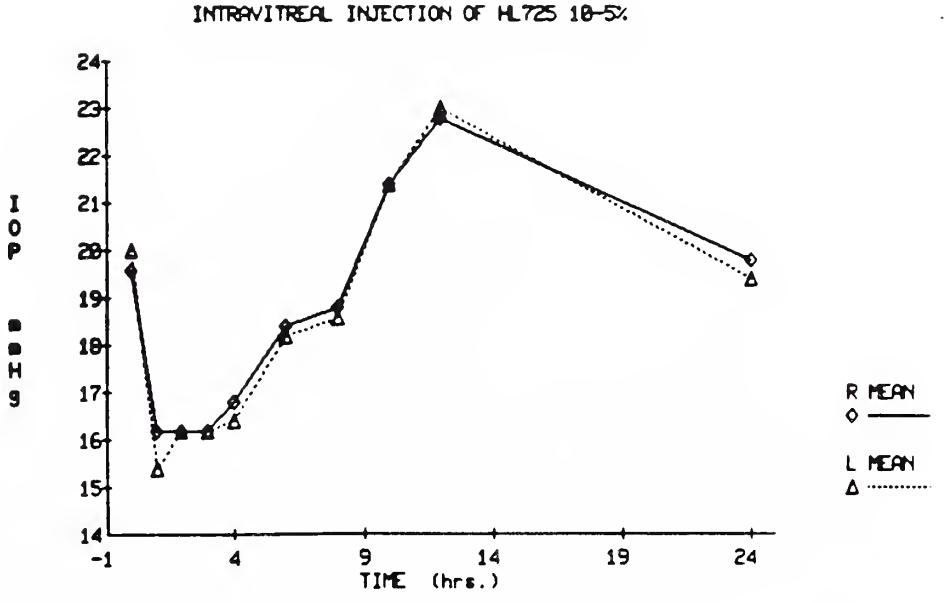


Figure 18: IOP vs. Time for eyes treated with intravitreal injection of HL725 10-5 % (final intravitreal concentration of 10-9 M) OD and of saline OS. (n=5)

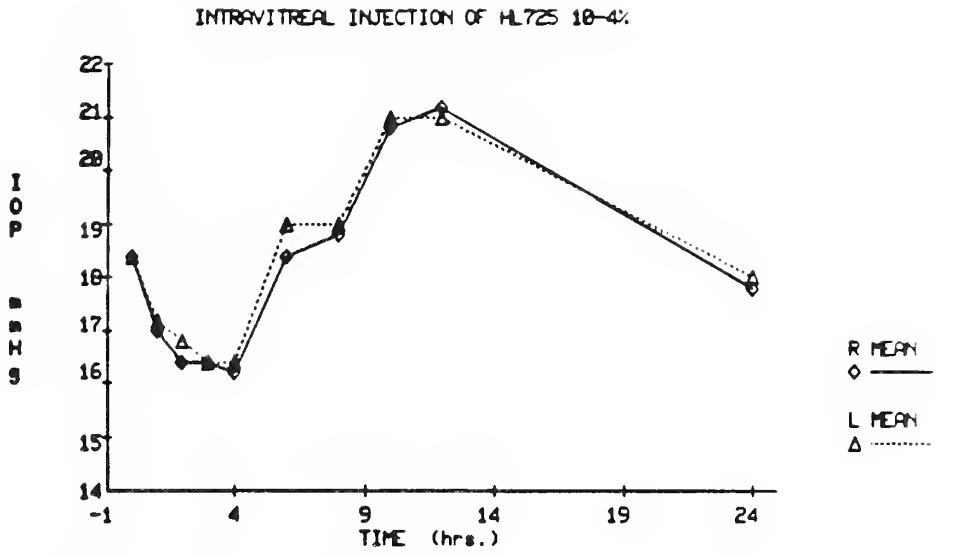


Figure 19: IOP vs. Time for eyes treated with intravitreal injections of HL725 10-4% (final intravitreal concentration of 10-8 M) OD and of saline OS. (n=5)

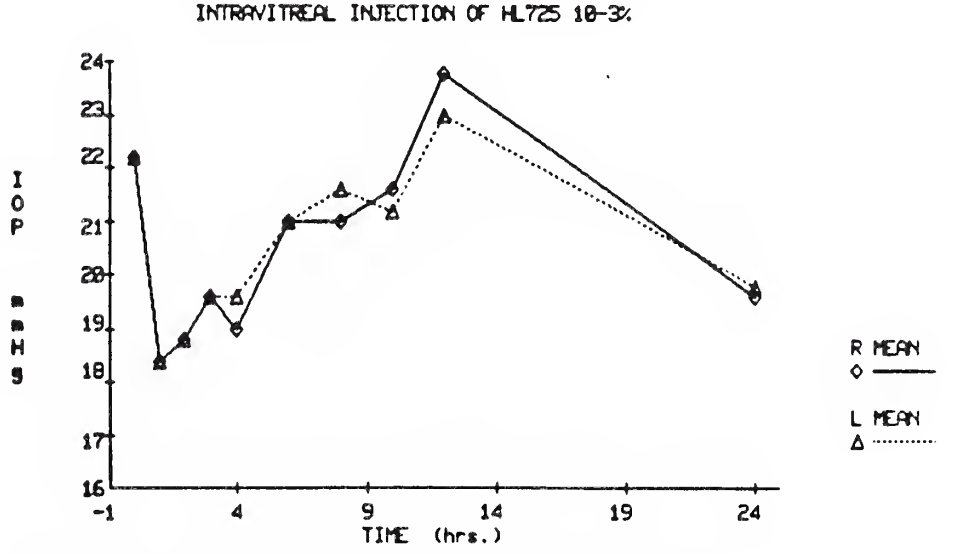


Figure 20: IOP vs. Time for eyes treated with intravitreal injections of HL725 10-3% (final intravitreal concentration of 10-7 M) OD and of saline OS. (n=5)

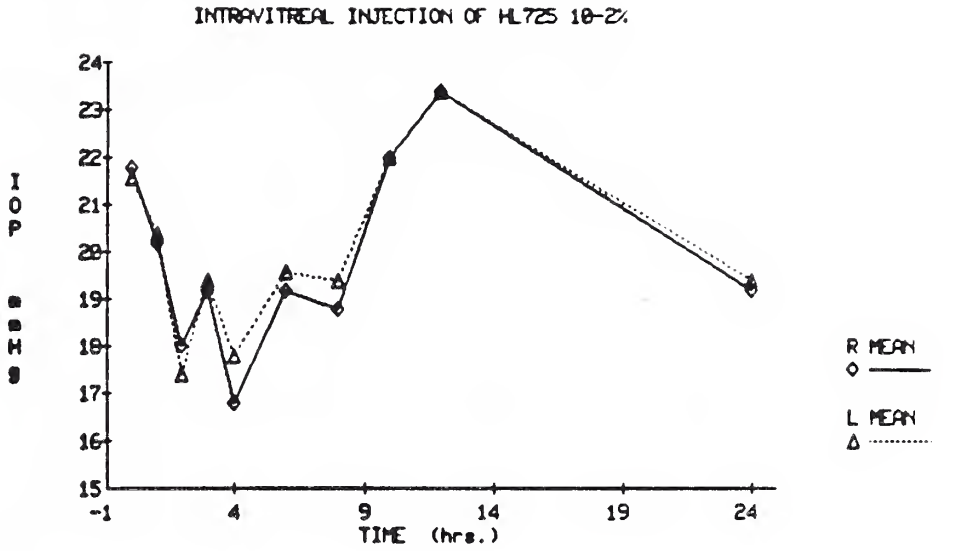


Figure 21: IOP vs. Time for eyes treated with intravitreal injections of HL725 10-2% (final intravitreal concentration of 10-6 M) OD and of saline OS. (n=5)

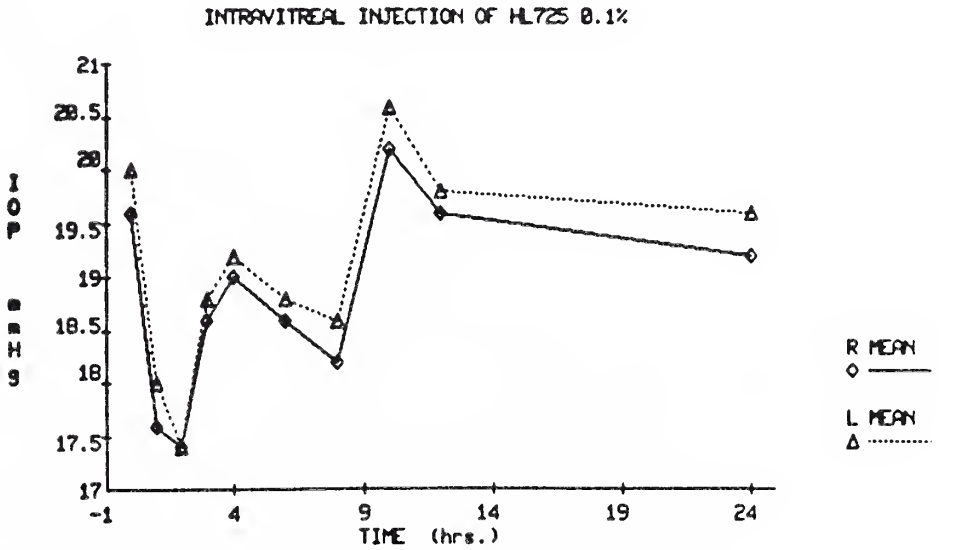


Figure 22: IOP vs. Time for eyes treated with intravitreal injections of HL725 10-1% (final intravitreal concentration of 10-5 M) OD and of saline OS. (n=5)

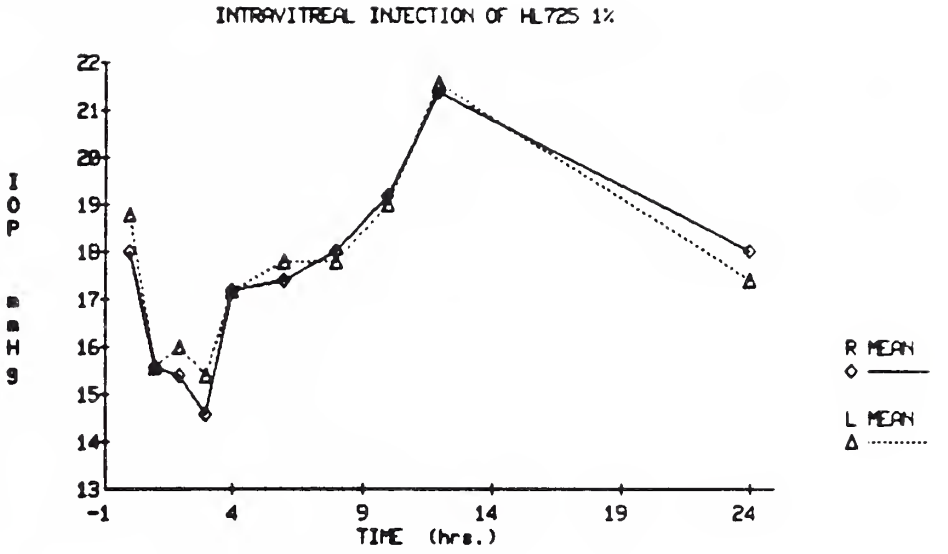


Figure 23: IOP vs. Time for eyes treated with intravitreal injections of HL725 1% (final intravitreal concentration of 10-4 M) OD and of saline OS. (n=5)

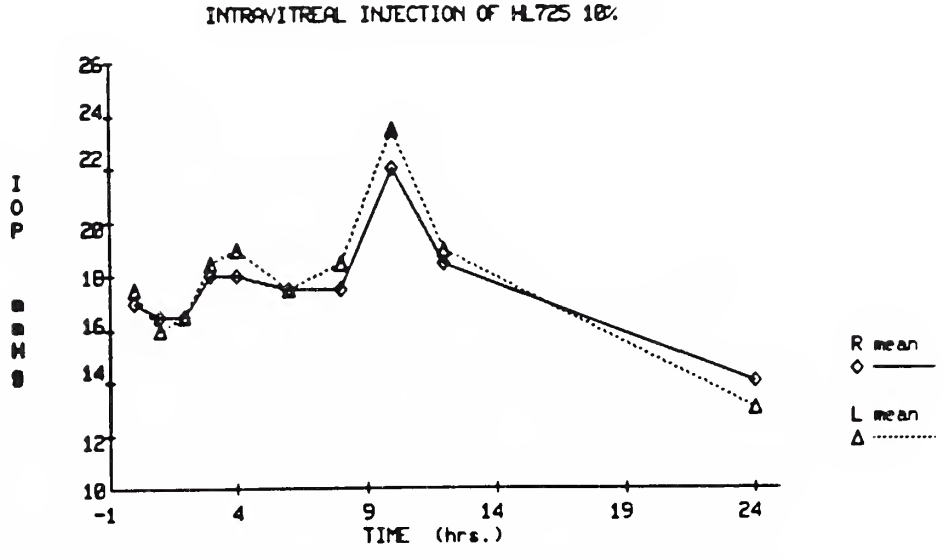


Figure 24: IOP vs. Time for eyes treated with intravitreal injections of HL725 10% (final intravitreal concentration of 10-3 M) OD and of saline OS. (n=2)

5. Topical Isoproterenol:

Topical applications of isoproterenol in concentrations ranging from 0.01% to 1.0% resulted in IOP decreases in the treated eye ranging from -3.4 to -5.0 mm.Hg. at 1 hour to 0 to -0.6 mm.Hg. at 12 hours. Effects of various concentrations of topical isoproterenol are represented by plots of (a) delta IOP (mean, n=5) vs. time for each concentration of isoproterenol and (b) [delta IOP x time] (area under the curve calculated for each delta IOP vs. time curve or IOP hours) vs. [log(concentration of isoproterenol)]. See figures 25 and 26.

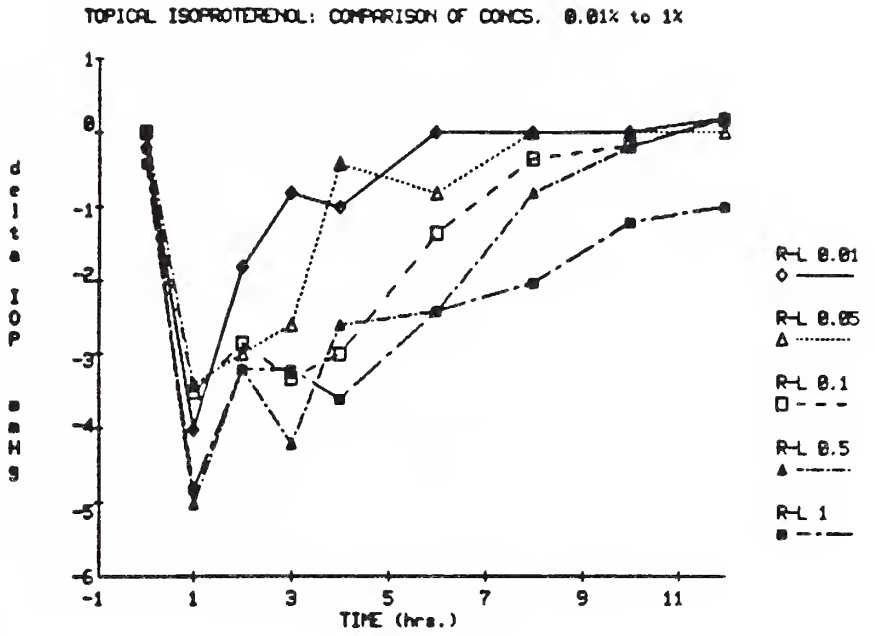


Figure 25: Delta (R-L) IOP vs. Time for topical applications of isoproterenol ranging in concentration between 0.01 and 1%. Values represent mean delta (R-L) IOPs for n=5.

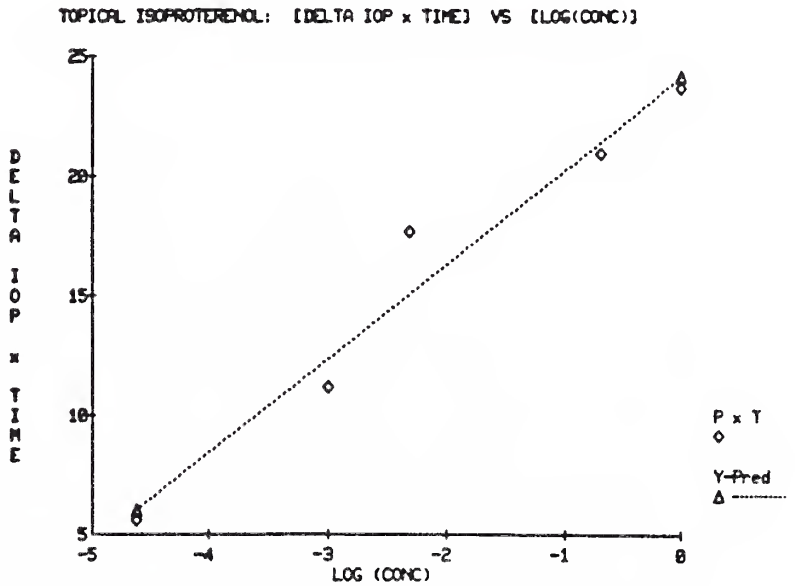


Figure 26: [Delta IOP x Time] vs. [Log(Conc.)] for topical applications of isoproterenol ranging in concentration between 0.01 and 1%. Values represent areas (under the curves) calculated from the curves of delta IOP vs. time for each concentration of isoproterenol.

6. Synergism Between HL725 and Isoproterenol:

a. Topical Isoproterenol 0.01% OU + Intravitreal HL725 OD:

Intravitreal injection of HL725 0.01% (final intravitreal concentration of 10^{-6} M) in the right eye (in comparison to intravitreal injection of saline in the left eye) resulted in modest potentiation of isoproterenol's hypotensive effect, with delta (R-L) IOP of -1.5 mm.Hg. at 4 and 6 hours ($p<0.05$, n=6). See figures 27 and 28.

Intravitreal injection of HL725 0.1% (final intravitreal concentration of 10^{-5} M) OD resulted in modest potentiation (longer term than that of 0.01% HL725) of isoproterenol's hypotensive effect. Delta IOP was -1.5, -2.2, and -1.0 mm.Hg. at 6, 10, and 12 hours, respectively ($p<0.05$, n=6). See figures 29 and 30.

b. Topical Isoproterenol 0.1% OU + Intravitreal HL725 OD:

Intravitreal injection of HL725 0.1% (final intravitreal concentration of 10^{-5} M) OD (incomparison to saline OS) appeared to cause a modest potentiation of isoproterenol's hypotensive effect, although with n=3 none of the differences of right and left IOP (delta IOPs) were statistically significant. See figure 31.

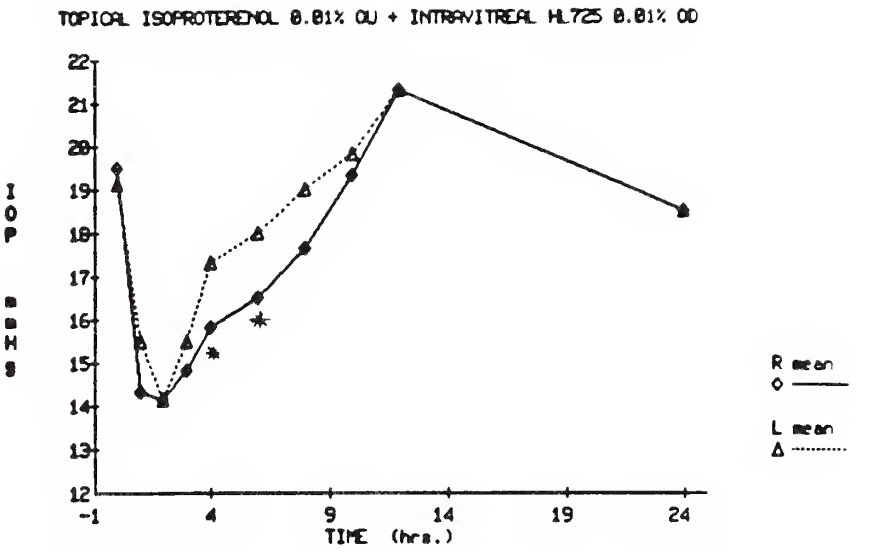


Figure 27: IOP vs. Time for topical applications of isoproterenol 0.01% OU plus intravitreal injection of HL725 0.01% (final intravitreal concentration of 10^{-6} M) OD and of saline OS. Values represent mean IOPs (n=6). *Significant at $p<0.05$ by paired t-test (two-tailed).

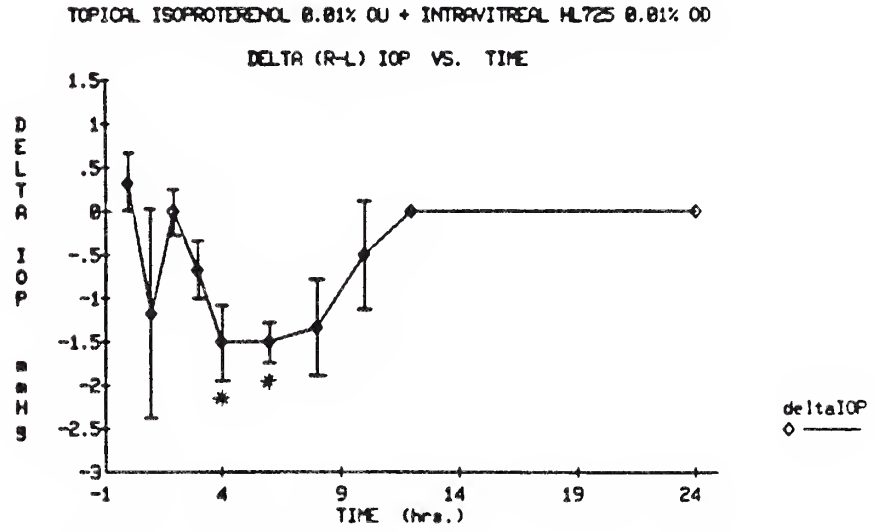


Figure 28: Delta (R-L) IOP vs. Time for topical applications of isoproterenol 0.01% OU plus intravitreal injection of HL725 0.01% (final intravitreal concentration of 10^{-6} M) OD and of saline OS. Values represent mean delta IOPs $\pm$ SEMs (n=6). *Significant at $p<0.05$ by two-tailed t-test.

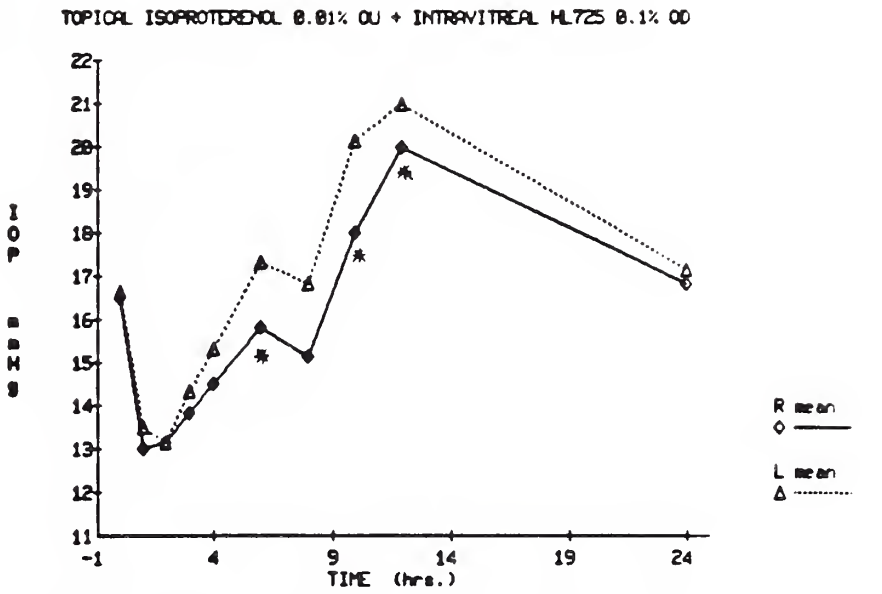


Figure 29: IOP vs. Time for topical applications of isoproterenol 0.01% OU plus intravitreal injections of HL725 0.1% (final intravitreal concentration of 10^{-5} M) OD and of saline OS. Values represent mean IOP (n=6). *Significant at $p<0.05$ by paired t-test (two-tailed).

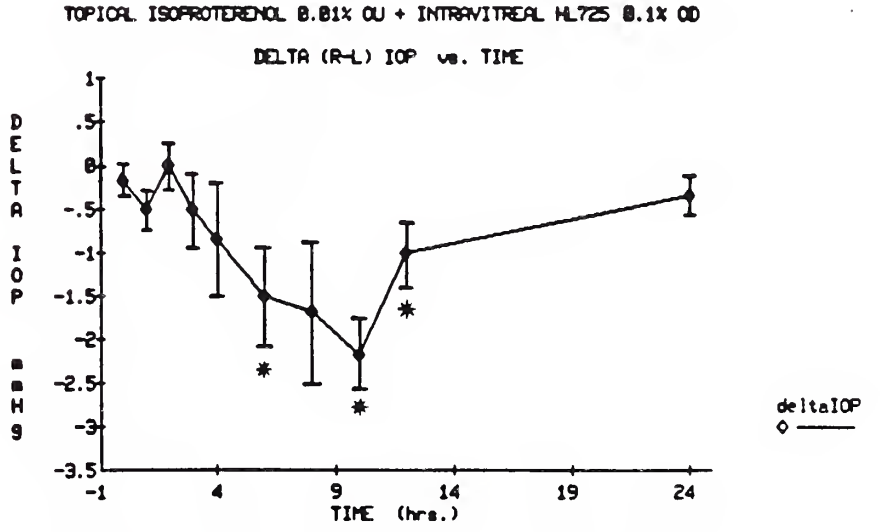


Figure 30: Delta (R-L) IOP vs. Time for topical applications of isoproterenol 0.01% OU plus intravitreal injections of HL725 0.1% (final intravitreal concentration of 10^{-5} M) OD and of saline OS. Values represent mean delta IOPs $\pm$ SEMs (n=6). *Significant at $p<0.05$ by two-tailed t-test.

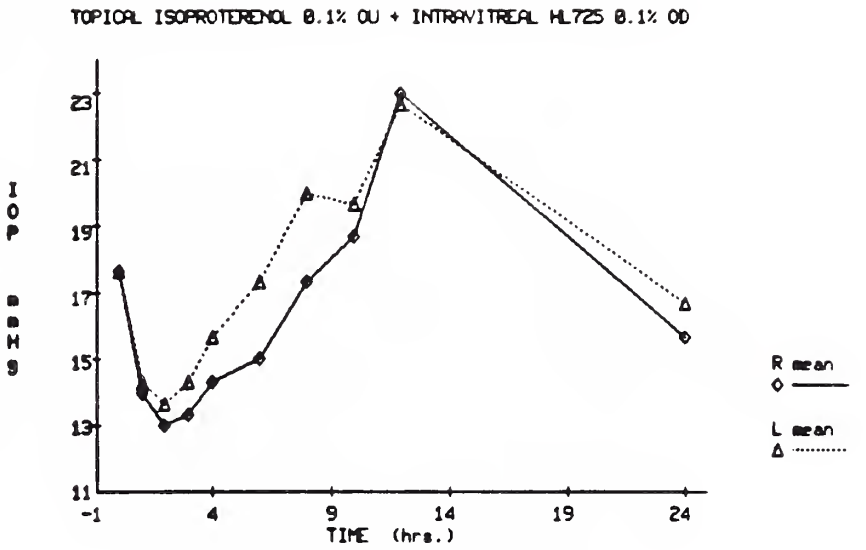


Figure 31: IOP vs. Time for topical applications of isoproterenol 0.1% OU plus intravitreal injections of HL725 0.1% (final intravitreal concentration of 10^{-5} M) OD and of saline OS. Values represent mean IOPs (n=3).

B. Rabbit Ciliary Process Cyclic Nucleotide Stimulation:

1. Ciliary Process cAMP Stimulation:

Ciliary process cAMP stimulation was greater in the presence than in the absence of HL725 at all concentrations of HL725 between 10^{-7} and 10^{-3} M. Stimulation of cAMP (cAMP level in the presence of HL725/basal cAMP level) was greatest at HL725 10^{-4} M. concentration. See figure 32.

2. Ciliary Process cGMP Stimulation:

Under the procedure conditions, preliminary studies of ciliary process cGMP stimulation found no detectable cGMP in the absence of HL725. Ciliary process cGMP was detectable at all concentrations of HL725 between 10^{-7} and 10^{-3} M. See figure 33.

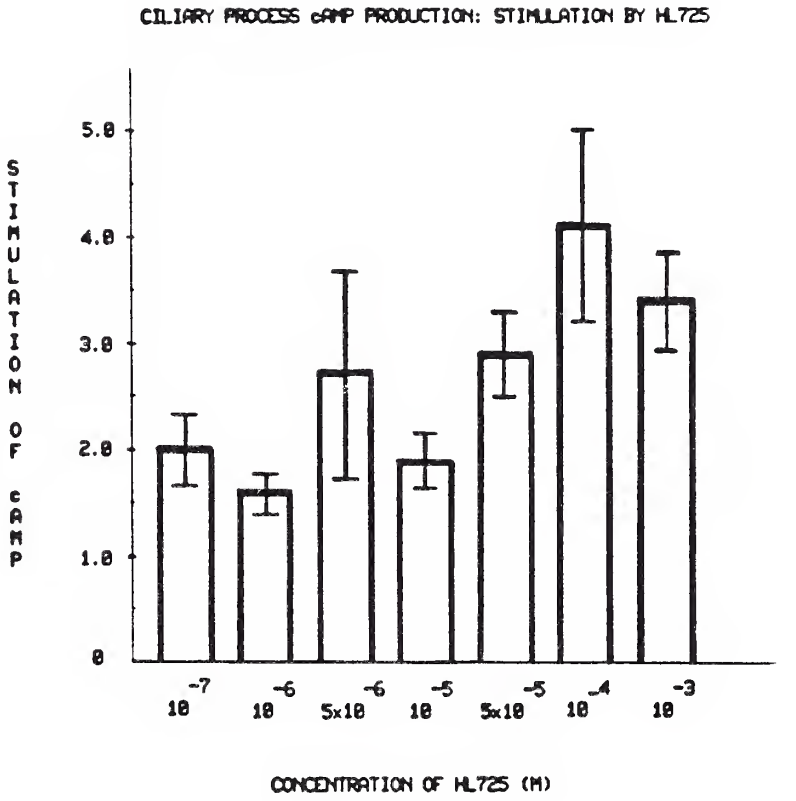


Figure 32: Ciliary Process cAMP Production: Stimulation of cAMP vs. Concentration (M) of HL725. Stimulation of cAMP equals cAMP (concentration at respective HL725 concentration)/ cAMP (concentration at zero M. HL725) or cAMP production/basal cAMP. Values represent means $\pm$ SEMs for n=6.

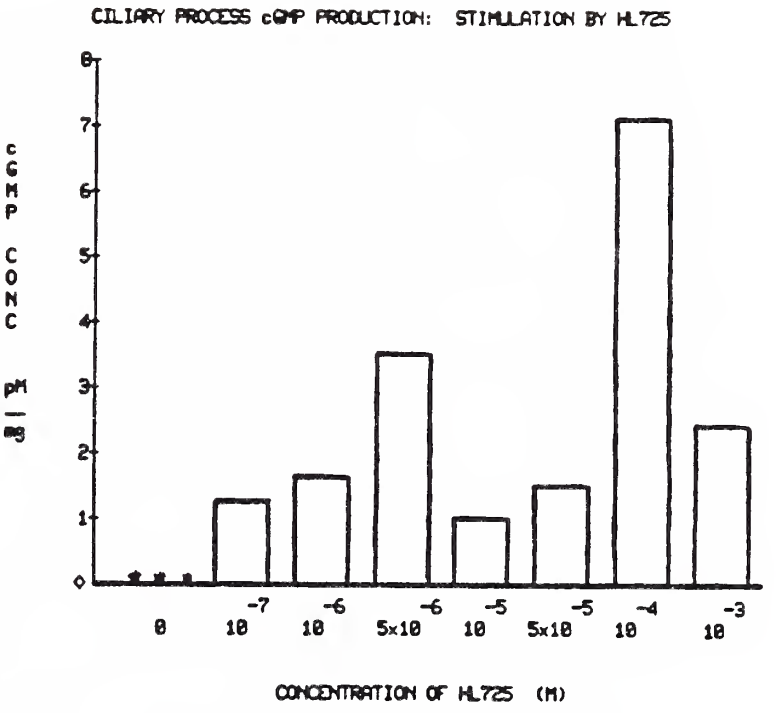


Figure 33: Ciliary Process cGMP Production: Stimulation by HL725. Production of cGMP (pM. cGMP/mg. protein) vs. Concentration (M) of HL725. ***cGMP concentration was undetectable by the RIA under the procedure conditions.

IV. DISCUSSION:

A. Pneumatonometer Calibration for Rabbit Eyes:

The applanation pneumatonometer consists of a floating tip sensor on a piston with a frictionless pneumatic bearing which allows the sensor to float on a cushion of gas (supplied by a compressed gas source) [Walker & Langham, 1975]. Application of the tonometer tangential to the cornea results in a displacement of the sensor membrane, an increase in the gas pressure, subsequent flattening of the corneal surface, and an inward spreading of the flattening across the corneal layers until the inward-acting force (F_1, pneumatic force) is balanced by the outward-acting force (F_2, intraocular force) [Durham et al., 1965]. The sensor is designed such that the force applied to the diaphragm from the gas pressure in the chamber (F_1) is equal to the force applied to the diaphragm by the pressure of the eye (F_2) and such that the sensor measures IOP when the outer coats of the eye are flattened (applanated) by contact. Pneumatic applanation tonometry is described by a function relating sensor P (P_1) to IOP (P_2):

$$2Rt/A^2 = (P_1/P_2)(\ln \Psi) + (1-\Psi),$$

where: R = corneal radius of curvature;

t = corneal deflection from an applanated configuration at the sensor edge;

A = jet tube radius;

P_1/P_2 = ratio of sensor pressure (P_1) to IOP (P_2); and

Ψ = factor dependent on geometric relationship of cornea and sensor during applanation (affected by pressure ratio and probe geometry).

Rearranging the above function:

$$P_1 = \{[2Rt/A^2 - (1-\Psi)](P_2)\}/\ln\Psi,$$

one can see that the measured intraocular pressure detected by the sensor (P_1) is dependent not only on the actual intraocular pressure and probe geometry but also on corneal radius of curvature (R) and other corneal properties (e.g.: thickness) affecting the deflection (t) of the cornea from the sensor edge during applanation. The slope of a graph of measured IOP (sensor or tonometer pressure, P_1) versus actual IOP (P_2) would reflect the corneal properties (radius of curvature and thickness) as well as probe geometry and tube radius.

The Digilab 30-D applanation pneumatonometer has been calibrated for the human eye, whose anterior corneal surface has a normal average radius of curvature equal to 7.8 mm. [Newell, 1982]. Because the applanation pneumatonometer was used to determine IOP of rabbits, whose corneal radius of curvature is dependent on size and age and has a normal average variously reported between 7.0 and 8.25 mm. [Prince, 1964], it was necessary to calibrate the pneumatonometer for rabbits similar in size to those used in the later studies.

The calibration curves for rabbit eyes demonstrated a direct relationship between measured IOP (P_1) and actual IOP (P_2), with mean slopes of calibration curves under closed stopcock conditions not significantly different from 1.0. It is apparent from these calibration data that the corneal properties of these rabbit eyes under normal conditions did not result in a relationship between P_1 and P_2 different from that found by calibration of the tonometer for human eyes.

In the calibration procedure with the stopcock open, any fluid displaced by corneal displacement during applanation tonometry would flow through the cannula back towards the reservoir, preventing any increase in IOP and therefore maintaining the IOP at the set manometric level. With the stopcock closed the conditions of the normal eye are reproduced, and any corneal indentation results initially in a slightly higher IOP (as intraocular fluid displacement causes increased IOP) and later in a slightly lower IOP (as pneumatonometer force causes egress of intraocular fluid through the outflow channels) [Durham et al., 1965; Langham & McCarthy, 1968]. Theoretically applanation tonometry, unlike indentation tonometry, causes minimal displacement of the cornea and intraocular fluid and therefore only small changes in IOP [Durham et al., 1964; Langham & McCarthy, 1968]. If corneal indentation is small enough the pressure measurement should be independent of ocular rigidity and the pressure-volume relationship of the eye [Langham & McCarthy, 1968].

Early studies of applanation pneumatonometry indicated that the IOP measurement could be taken before IOP was altered by any corneal

indentation or applanation pressure and that a close correlation between tonometric and manometric IOPs could be obtained under open and closed stopcock conditions [Durham et al., 1965]. With a rigid sensor tip (without pneumatic bearing) the pressure difference between closed and open stopcock procedures was 1 mm.Hg. at an IOP of 15 mm.Hg. and 2 mm.Hg. at an IOP of 20 mm.Hg. (with corresponding volume displacements of 2.2 μl. and 0.8 μl., respectively) [Langham & McCarthy, 1968]. With an air-bearing floating tip the pneumatic force (approximately 2 g.) causes an additional pressure difference of 1-2 mm.Hg. [Walker & Langham, 1975]. If the tonometer is acting as a true applanating system, corneal indentation and IOP change should be small enough that IOP measurements under open and closed stopcock conditions should be equal [Langham & McCarthy, 1968].

In these calibration experiments there was a significant difference in the slopes obtained with open and closed stopcocks. These results suggested that the applanation tonometer was not a true applanating system and was causing a detectable (though small) amount of corneal indentation. In this case it would be important to convert tonometer reading to actual IOP (manometer pressure) using the calibration curve for conditions analagous to the normal eye (i.e.: closed stopcock), but because the slope of this curve, 0.98 ± 0.02, was not significantly different from 1.0 it was unnecessary to convert the recorded IOP measurements to a value corrected for the rabbit eyes.

Based upon the observation that the pneumatic tonometer caused indentation significant enough to result in a difference between closed

and open stopcock measurements, one would suspect that the pressure measurements would not be completely independent of ocular rigidity and the pressure-volume relationship of the eye. Although the measurements would give no direct estimate of ocular rigidity [Sears, 1966] they would be affected by the ocular rigidities of the eyes. Differences in odular rigidity (caused by post-mortem changes as well as differences already existent in vivo) could result in different calibration curves. In this case no difference was observed between calibration curves of enucleated eyes and eyes in their orbits used within a few hours post-mortem.

B. IOP Circadian Rhythm:

The rabbits used in these studies were not kept in as well-controlled an environment or entrained as long as the rabbits of the two previously reported reproducible rabbit IOP circadian rhythm models. Nevertheless, they exhibited a remarkably consistent IOP circadian rhythm. Rabbits entrained to the light:dark cycle for only two days exhibited a circadian rhythm similar to that of rabbits kept in the same environment between 1 day and several weeks. Entrainment of a circadian rhythm or resetting/phase-shifting of a circadian pacemaker can be effected by as little as one brief exposure to light during a receptive period of the phase-response curve, but entrainment or resetting of a circadian pacemaker can require as much as several days to weeks exposure to a new light:dark cycle [Moore-Ede et al., 1982 & 1983]. The rabbits used in this series of experiments may have been kept in an en-

vironment with a similar light:dark environment prior to their delivery at Yale or may have required merely brief exposure to the light/dark cycle in the new environment for entrainment of the IOP circadian rhythm.

The similarity between the two curves, representing the IOP circadian rhythms of (1) a group of rabbits used specifically to document the IOP circadian pattern and of (2) several rabbits used for HL725 studies over the course of several months, suggests that the possible variation in the rabbits' environment (exposure to small amounts of light during dark periods, changes in social cues by different investigators entering the rooms at different times on different days, and other possible changes which might occur in a communal housing facility) were not significant enough to cause obvious changes in the rabbits' IOP rhythm.. The light:dark cycle cue and perhaps the social cues of animal care facility workers arriving and leaving the area at the same time each day were consistent enough to maintain a reproducible IOP rhythm. The small amount of light to which the rabbits were exposed during the early portion of the dark period would have been expected to cause a phase delay in the circadian rhythm [Moore-Ede et al., 1982& 1983] if any effect at all. The light exposure may have caused no effect because its intensity and duration were insufficient to change the circadian rhythm, because it fell during a time of instability on the phase-response curve [Moore-Ede, 1982], or because other environmental cues (e.g.: social cues of workers arrival and departure at the same time each day) provided additional strength to the light cue.

The rabbit IOP circadian rhythm in this series of studies was similar to the circadian rhythms of the rabbit models of Rowland et al. [1981] and Gregory et al. [1985], although the shape and amplitude of the curve differed in some respects. In comparison to the rabbit IOP pattern of Rowland et al [1981] and of Gregory et al. [1985], the circadian curve had a smaller amplitude (6-7.4 mm.Hg. versus 12-13 mm.Hg. and 7.4-8.5 mm.Hg.). In this study measurements were taken less frequently during the dark period, and it is possible that a larger amplitude might have been seen if an additional reading had been taken between 12 and 16 hours circadian time. The small amount of light entering the animal rooms from emergency lighting in the hallways might have caused a diminished IOP amplitude by decreasing the difference between light and dark intensity. A greater intensity of overhead illumination or a dawn and dusk transition of illumination [Moore-Ede et al, 1982] might result in a larger amplitude of IOP change.

Despite the minor differences between the IOP pattern and that of the other two models, in the environment described the rabbits exhibited a reproducible IOP circadian rhythm with IOP lower during the light period and higher during the dark period. Rabbits in this environment could be used to study the effects of various agents on IOP during different periods of the circadian rhythm and the mechanisms effecting the IOP circadian pattern. To alleviate any possible changes of the rhythm by light exposure during the dark period and to facilitate more frequent IOP measurement in the dark, a long-wavelength red light could be employed during reading in the dark phase of the cycle.

Of what importance is the circadian rhythm in ophthalmologic research? Almost all physiologic variables exhibit circadian rhythms, and any investigation of mechanisms controlling or drugs affecting a physiologic variable must take into consideration the possible effects of circadian variation in that variable and other systemic variables. The circadian system is believed to consist of central pacemakers or primary oscillators, secondary oscillators as well as passive elements in peripheral tissues, transducers or receptors which transform environmental into biologic cues, and mediators which transmit temporal information between transducers, primary oscillators, secondary oscillators, and passive elements. All portions of this system need be considered in an analysis of circadian rhythms. Circadian rhythmicity appears to occur at all physiologic levels, with plasma and anterior chamber volume [Mapstone & Clark, 1985], plasma hormone level, receptor number and affinity [Titanchi et al., 1984], intracellular enzyme inducibility [Moore-Ede et al, 1982], and even cellular content of cAMP and cGMP [Murakami & Takahashi, 1983; Schwarz et al., 1984] and guanyl cyclase activity [Cannela & Vesely, 1984] exhibiting circadian rhythms. Drug effectiveness and toxicity is affected by circadian rhythms of drug absorption, metabolism, excretion, plasma protein binding, and plasma dilution degree, and by circadian variation of tissue susceptibility [Moore-Ede et al., 1982].

Circadian variations of mediator secretion and tissue susceptibility may be some of the more important factors of pharmacologic effectiveness. In the example of systemic hypertension, beta-blockers are known to be effective treatment, but during the period between 0600 and

1000 hrs. when the most rapid rise of arterial blood pressure occurs beta-blockers appear not to lower blood pressure possibly because of an increase in sympathetic activity and alpha-adrenergically mediated peripheral arteriolar constriction [Raftery & Carrageta, 1985] on which beta-blockers would have no effect. The differences of effectiveness of epinephrine, timolol, and acetazolamide in changing aqueous humor formation at different circadian times may be caused by circadian rhythms of tissue susceptibility at the receptor or intracellular level or of endogenous hormone secretion. The mechanism controlling IOP circadian changes may be adrenergically mediated [Gregory et al., 1985], and one possible mechanism controlling IOP may involve an adenyl cyclase-receptor complex. The effects of alpha- and beta-adrenergic agents may be different at different circadian times because of circadian rhythms of plasma hormone and catecholamine levels and of tissue susceptibility (including receptor level and affinity, intracellular enzyme inducibility, cellular content of cyclic nucleotides, and other factors). Obviously it will be important in the future to investigate the circadian rhythms of physiologic, pharmacologic, and biochemical properties of the eye as well as the oscillator, transducer, and mediator components of the circadian system in order to elucidate the mechanisms controlling IOP. Preliminary studies are being performed on rabbit models prior to human investigations.

C. Intracellular Messengers, a Phosphodiesterase Inhibitor, and IOP:

Following the discovery of cAMP [Cook et al., 1957; Sutherland & Rall, 1957] and the report of its action as the intracellular mediator of epinephrine's and glucagon's glycogenolytic effect in the liver [Sutherland & Rall, 1960], adenyl cyclase and cAMP were found in all animal species and all tissues [Perkins, 1973] and cAMP was shown to be the intracellular mediator of the effects of a variety of hormones on a variety of tissues [Sutherland et al., 1968; Butcher et al., 1970; Jost & Rickenberg, 1971; Rall, 1982]. cAMP was designated the ubiquitous second messenger, and a simple model of the cAMP-mediated system was characterized by the series of events in which a hormone (first messenger) stimulated adenyl cyclase to catalyze the conversion of ATP to cAMP (second messenger) which then caused a physiologic response by activation of protein kinases [Walsh et al., 1968; Krebs & Preiss, 1975; Beavo & Mumby, 1982]. The intracellular message was terminated by degradation of cAMP to 5'-AMP by phosphodiesterase [Sutherland et al., 1968].

Since the proposal of the model of the second messenger system, theories of hormonal regulation and models of intracellular metabolism have become increasingly complex with the discovery of other cyclic nucleotides and intracellular messengers and of other regulatory proteins and enzymes. The model is not yet complete and the role of many of the components of the model is not clear, but the picture includes stimulatory and inhibitory membrane receptors, stimulatory and inhibitory guanine nucleotide binding proteins, adenyl and guanyl cyclases

(membrane-associated and cytosolic), multiple forms of cyclic nucleotide phosphodiesterases (membrane-associated and cytosolic), calmodulin and other regulatory proteins and ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, and Na^{+}).

The plasma membrane contains (or has associated with it) three protein components of the adenyl cyclase-receptor system: receptors, nucleotide regulatory proteins, and catalytic components of adenyl cyclase. Stimulatory and inhibitory receptors are coupled to adenyl cyclase. Stimulatory receptors (R_s) include beta-adrenergic [Hekman et al., 1984; Caron et al., 1985], adenosine (A_2) [Daly, 1985], dopamine (D_1) [Cote et al., 1985], LH and other hormones [Ross & Gilman, 1980]. Inhibitory receptors (R_i) include alpha $_2$-adrenergic [Lefkowitz et al., 1984; Caron et al., 1985; Mitrius & U'Prichard, 1985], adenosine (A_1) [Daly, 1985; Phillis & Barraco, 1985], muscarinic cholinergic [Harden et al., 1985], dopamine (D_2) [Cote et al., 1985], opiate ( ), various prostaglandins [Jakobs et al., 1984], and perhaps progesterone [Sadler & Maller, 1985]. The binding of hormones (and neurotransmitters) to specific receptors (R_s or R_i) initiates the hormonal response [Birnbaumer & Iyengar, 1982].

"G" or "N" proteins, the guanine-nucleotide-binding regulatory proteins, transfer information from receptors to their effector molecules. The three N proteins known at present are N_s and N_i (the stimulatory and inhibitory N proteins which transfer information from stimulatory and inhibitory receptors, respectively, to the catalyst of adenyl cyclase) and transducin (which transfers information

from retinal rod outer segment rhodopsin to cGMP-specific phosphodiesterase) [Smigel et al., 1984 & 1985]. Both N_s and N_i are activated by binding and hydrolysis (by GTPase), respectively, of GTP with a change in the conformation of the alpha, beta, and gamma subunits of the N protein [Hildebrandt et al., 1985; Cooper et al., 1985]. Activation of N_s and N_i requires Mg^{2+} or another divalent cation and is inhibited by sodium ion [Klee et al., 1984; Jakobs et al., 1984]. The N_s-GTP complex activates the adenyl cyclase catalyst by the release of the alpha subunit of N_s. The mechanism of N_i-GTP complex inhibition of the adenyl cyclase catalyst is not yet known [Jakobs et al., 1985].

The catalytic site of adenyl cyclase is the third component of the membrane complex. Activation or inactivation of the catalyst is achieved by N_s or N_i, respectively, after the binding of a hormone to R_s or R_i, respectively. Activation of the catalyst results in the conversion of ATP to cAMP and therefore the increase in intracellular cAMP concentration. Conversely, inactivation of the catalyst inhibits the production of cAMP from ATP [Rall, 1982; Birnbaumer & Iyengar, 1982; Smigel et al., 1985].

The adenyl cyclase system appears to exist predominantly in the plasma membrane (although its existence in subcellular membranous structures has not been excluded [Perkins, 1973]) and to exert its physiologic effects in various tissues by hormone-stimulated protein kinase activation [Beavo & Mumby, 1982}. The location and function of the guanyl cyclase system are not as clear [Rall, 1982]. Guanyl

cyclase has been found in particulate and soluble fractions [Rall, 1982] in multiple forms [Mittal & Murad], and only a few physiologic functions have been shown definitively to be regulated by cGMP [Walter, 1984]. Soluble guanylate cyclase is activated not by an N_s protein but by fatty acids, free radicals, and redox agents which are believed to interact directly and reversibly with the enzyme [Murad et al., 1981]. The soluble guanyl cyclase has free sulfhydryl groups and exists in a heme-associated and heme-free form [Bohme et al., 1984; Ignarro et al., 1984]. Because hormones have been shown to increase intracellular cGMP concentration but not to stimulate cell-free guanyl cyclase preparations it has been postulated that hormonal effects on cGMP are a result of a chain of reactions (stimulated by a hormone-receptor interaction) involving Ca^{2+} - and oxygen-dependent steps and eventual guanyl cyclase activation [Bohme et al., 1984]. The particulate guanyl cyclase from sea urchin spermatozoa has been purified, but no mammalian guanyl cyclase has yet been purified [Garbers & Radany, 1981] despite the fact that it has been found associated with plasma membranes and subcellular membranes [Murad et al., 1979]. Activation of guanyl cyclase is known to require Mn^{2+} and GTP [Goldberg & Haddox, 1977]; further characterization of particulate guanyl cyclase properties awaits purification of a mammalian form.

In most tissues the amount of guanyl cyclase and cGMP is 1/10 to 1/100 that of adenyl cyclase and cAMP [Goldberg et al., 1973; Mittal & Murad, 1982]. A few specialized tissues (e.g.: retinal rod outer segment [Farber, 1982]) contain significantly more guanyl cyclase and cGMP than adenyl cyclase and cAMP. Early studies of the regulatory function

of cGMP compared and contrasted its physiologic effects with those of cAMP and resulted in a "Yin Yang hypothesis" of cellular regulation accomplished by opposing effects of cGMP and cAMP [Goldberg et al., 1973; Goldberg et al., 1975]. Subsequent studies indicated that cGMP had effects opposing as well as mimicking those of cAMP [Goldberg & Haddox, 1977; Murad et al., 1979]. The protein kinase specific for cGMP appears to be located primarily in the cytoplasm and possibly subcellular organelles [Kuo & Shoji, 1982; Lincoln & Corbin, 1983]. Studies of this kinase and cGMP stimulation have implicated cGMP in regulatory functions including negative feedback mediation of Ca^{2+} entry in cells, vascular smooth muscle relaxation, platelet aggregation, and intestinal secretion [Lincoln & Corbin, 1983; Murad et al., 1979]. cGMP apparently has regulatory functions mediated by other binding proteins besides protein kinase. A cGMP-binding protein phosphodiesterase is the more abundant cGMP-binding protein in platelets and rod outer segments, suggesting a regulatory role for cGMP in platelet aggregation and visual transduction of rod photoreceptors. A cGMP-binding protein-cAMP-phosphodiesterase is widely distributed in tissues and suggests a role for cGMP in controlling cAMP levels by stimulating a cAMP phosphodiesterase [Lincoln & Corbin, 1983].

The level of intracellular cyclic nucleotides, after synthesis has occurred, can be controlled by extrusion [Goldberg et al., 1973] or efflux from the cell, subcellular sequestration, or degradation by phosphodiesterases [Appleman et al., 1982]. At least three forms (cAMP, cGMP, and cyclic nucleotide) of phosphodiesterase occur in different proportions in different tissues, and probably in different subcellular

locations. (More specific forms of phosphodiesterase occur only in certain specialized tissues, e.g.: light-activated cGMP phosphodiesterase in rod outer segment.) All forms of phosphodiesterase have been located in soluble and particulate cell fractions, although the cAMP and cGMP phosphodiesterases appear to be primarily particulate whereas the cyclic nucleotide phosphodiesterase is primarily soluble or cytosolic [Appleman et al., 1982]. The high affinity (low K_m) cAMP phosphodiesterase is inhibited by cGMP [Appleman et al., 1973]. The cGMP phosphodiesterase (or "high K_m" cyclic nucleotide phosphodiesterase) is stimulated by Ca^{2+}-calmodulin [Krinks et al., 1984]. The cyclic nucleotide phosphodiesterase is allosterically activated by low concentrations of cGMP [Wells & Hardman, 1977; Erneux et al., 1984]. A fourth phosphodiesterase, which is a calcium-independent form of the cGMP or "high K_m" cyclic nucleotide phosphodiesterase, also has been identified [Manganiello et al., 1984].

The cyclic nucleotides are not the only second messengers. Calcium is "a fundamental regulator of function in all cells" [Gill, 1985] and may in fact be a primary messenger with the cyclic nucleotides serving as secondary messengers [Berridge, 1984]. The discovery of calmodulin in 1970 [Cheung & Storm, 1982] and of calcium-dependent protein phosphorylations [Schulman, 1982] was followed by the characterization of multiple regulatory activities of calmodulin, including activation of the high K_m cyclic nucleotide (or cGMP) phosphodiesterase, activation and inhibition of adenyl cyclase, activation of some forms of guanyl cyclase, regulation of phosphorylase kinase, calcium-dependent protein kinase, calcium-ATPase and other enzymes, and regulation of neuro-

transmitter release and cytoskeleton organization [Cheung & Storm, 1982; Manalan & Klee, 1984]. The interaction between calcium and cyclic nucleotide enzymes (and the observation of cellular metabolism regulation by changes in concentrations and subcellular localizations of calcium and the cyclic nucleotides) led some authors to speculate that calmodulin, as the protein mediating calcium's effects, might be the coordinating regulator of calcium and cyclic nucleotide effects and therefore of cellular function [Watterson et al., 1984].

Manalan and Klee [1984] pointed out that calmodulin was a "ubiquitous protein" and that calcium was an intracellular messenger as important as cAMP. Berridge [1984] in an analysis of the interactions between calcium and the cyclic nucleotides concluded that calcium was the primary activator of cellular processes and that cAMP acted only indirectly as a modulator of calcium-dependent reactions. cAMP can affect calcium influx by cAMP-dependent phosphorylation [Gill, 1985] and can alter calmodulin's effects by cAMP-dependent phosphorylation of calmodulin's target proteins [Manalan & Klee, 1984]. cAMP functions as a modulator by changing the clcium signal generation and calcium-dependent processes [Berridge, 1984]. Methods of intracellular communication demonstrate calcium's role as the primary second messenger and the cyclic nucleotides' role as secondary second messengers. Stimulation of calcium receptors results in increased intracellular calcium, phosphoinositide hydrolysis, C-kinase activation, and arachidonic acid release. Guanyl cyclase is activated in the calcium-receptor-stimulated cell, and prostaglandin, prostacyclin, thromboxane, and leukotriene products are formed from the cyclooxgenase and lipoxygenase

pathways. The eicosanoids act as local hormones, diffusing to other cells and activating adenyl cyclase and affecting calcium levels (e.g.: the local hormone effect of PGI_2 in platelets is increased cAMP, inhibition of platelet aggregation, and subsequent inhibition of calcium receptors) [Berridge, 1984].

What are the components of the calcium messenger system, and how is it believed to act? The intracellular calcium level is a function of calcium movement between the cell and the large extracellular pool and calcium subcellular sequestration in endoplasmic reticular and mitochondrial membranes. The intracellular calcium levels are controlled by the calcium channel mediating calcium influx into the cell, calcium-ATPase mediating calcium efflux from the cell, and the sodium/calcium exchanger mediating movement of calcium both ways across the plasma membrane. The calcium-ATPase has a low pumping capacity but acts rapidly when stimulated by calcium-calmodulin, and the sodium/calcium exchanger has a high pumping capacity which is activated by calcium-calmodulin-dependent membrane-bound protein kinase. The calcium channel can be affected by cAMP-dependent phosphorylation resulting in an influx of calcium with increased cAMP levels [Carafoli, 1984]. Receptors that are distinct from adenyl cyclase receptors and are capable of altering intracellular calcium levels and calcium-dependent reactions have been shown to be affected by alpha $_1$, muscarinic, histaminic (H_1), serotonergic, and other agents. Binding to these receptors appears to stimulate a phospholipase C phosphodiesterase which cleaves polyphosphoinositol headgroups from phosphotidyl inositides (PI) [Gill, 1985].

The calcium messenger system has two branches [Rasmussen et al., 1984]: one which acts via amplitude modulation of calcium-regulated reactions and causes initiation of cellular response (to a hormone), and a second which acts via sensitivity modulation of calcium-sensitive phospholipid-dependent protein kinase (C-kinase) and causes a sustained cellular response. Binding of hormone to calcium receptors results in a cascade of reactions beginning with the hydrolysis of PI 4,5-biphosphate and/or PI 4-phosphate which causes activation of the two branches of the calcium messenger system. In the first branch the intracellular calcium level is increased abruptly by influx of calcium from the extracellular pool and release of calcium from subcellular pools. Increased intracellular calcium binds to calmodulin and activates calmodulin-regulated enzymes, with protein kinase activation resulting in phosphorylation of numerous cellular proteins and plasma membrane calcium pump activation resulting in lowered calcium levels by efflux and intramitochondrial sequestration. In the second branch the cell membrane diacylglycerol content is increased, C-kinase is bound to the plasma membrane and thereby activated, and C-kinase activation results in phosphorylation of other cellular proteins and a sustained cellular response (to the hormone) [Rasmussen et al., 1984]. Hormones and extracellular messengers regulate cell activity by increasing cytosolic calcium concentration. Calcium acts as a "universal intracellular messenger" through the calmodulin and C-kinase branches [Rasmussen et al., 1984]. cAMP can alter the action of calcium by affecting some of the calmodulin and C-kinase branch reactions.

Although calcium may be a more important second messenger than

previously realized it is apparent that both calcium and cyclic nucleotides serve as intracellular messengers regulating the physiologic effects of hormones and other extracellular messengers. The complex interactions between these intracellular messengers and with their target enzymes and proteins results in an overall cellular metabolism. Other roles and interactions for these and other intracellular messengers must exist, and further studies of cellular mechanisms and their components undoubtedly will lead to an even more complex picture of cellular metabolism and extra- to intracellular communication. Already other ions besides calcium, including chloride and sodium, have been implicated as regulators of cyclic nucleotide activity [Rodbell, 1984].

The numerous components of the cyclic nucleotide and interrelated calcium messenger systems allow for several modes of down-regulation and self-modulation. Some type of down-regulation of receptors, nucleotide regulatory proteins, and phosphodiesterases is known to occur. Persistent stimulation of beta-receptors causes a decrease in cAMP production and the physiologic effect. Beta-receptor desensitization can be homologous or heterologous, blunting the response of the receptor to a specific hormone or to all agonists, respectively. Homologous desensitization is not cAMP-mediated and may involve uncoupling of the receptor from adenyl cyclase or translocation of the receptor from the plasma membrane after receptor phosphorylation. Heterologous desensitization appears to involve receptor uncoupling and phosphorylation and possibly nucleotide regulatory protein phosphorylation. Receptor phosphorylation can be activated by cAMP-dependent kinase, calcium-sensitive C-kinase, and other protein kinases [Sibley & Lefkowitz, 1985;

Lefkowitz et al., 1984; Perkins et al., 1982]. Other receptors could be desensitized by mechanisms similar to homologous and heterologous beta-receptor desensitization.

Counterregulation of nucleotide protein adenyl cyclase activation is accomplished by N_s activating N_i as well as the adenyl cyclase catalyst [Jakobs et al., 1985]. Sodium ion apparently modulates adenyl cyclase activity by an action on N_i and perhaps R_i [Rodbell, 1984; Jakobs et al., 1985].

cAMP phosphodiesterase conversion to a less-active form by its own substrate cAMP could be considered yet another example of counterregulation [Robison et al., 1984]. Another form of down-regulation of hormone-stimulated physiologic effects occurs with activation of calmodulin-stimulated cyclic nucleotide phosphodiesterase during chronic beta-adrenergic stimulation [Manalan & Klee, 1984], and with decreasing the $K_{0.5}$ of calmodulin-stimulated phosphodiesterase for calmodulin in the presence of cyclic nucleotides [Krinks et al., 1984]. Stimulation of cAMP phosphodiesterase by cGMP may be a type of self-regulatory process in the cell.

In addition to being prime sites for counterregulation, phosphodiesterases appear to be the target of some hormones' primary effects. Muscarinic cholinergic receptor stimulation appears to exert its second messenger effect not only by activating the N_i protein but also by activating the calmodulin-stimulated phosphodiesterase [Harden et al., 1985]. Insulin appears to antagonize the effect of glucagon on the

liver by activating the cAMP phosphodiesterase [Housley et al., 1984; Appleman et al., 1984].

This picture of the complex second messenger system, with its multiple components, messengers, regulatory reactions, and counter-regulatory mechanisms, provides a background against which to consider phosphodiesterase inhibition and its effects. Phosphodiesterases degrade cAMP and cGMP and thereby bring an end to the intracellular hormonal message. In their multiple forms with unique modes of regulation (positive and negative cooperativity and calmodulin stimulation) they appear to provide the possibility of "finely controlled, multifaceted regulation of cyclic nucleotide metabolism" [Manganiello, 1984; Vaughan, 1981]. Phosphodiesterases should be able to monitor the intensity and duration of the intracellular message [Strada et al., 1984] and therefore the physiologic effect of the first messenger. Phosphodiesterase inhibitors selective for a particular form of phosphodiesterase theoretically would alter the concentration of a specific cyclic nucleotide in a tissue (depending upon the percentage and regulatory importance of each phosphodiesterase form in that individual tissue) and would have potential to elicit specific cellular responses [Weiss, 1975; Hidaka & Endo, 1984; Dudkin et al., 1983]. Phosphodiesterase inhibitors therefore would be able not only to mimic or potentiate the action of extracellular messengers (e.g.: hormones, neurotransmitters) [Wells & Kramer, 1981] but also to treat diseases for which altered cyclic nucleotide metabolism is responsible [Weiss & Hait, 1977].

The more well-known phosphodiesterase inhibitors, methylxanthines

and papaverine, have been used to increase cyclic nucleotide levels and to effect physiologic changes such as smooth muscle relaxation [Chasin & Harris, 1977]. These agents, like many other phosphodiesterase inhibitors, often cause nonspecific elevations of cAMP and cGMP in various tissues and often exert physiologic effects through actions other than phosphodiesterase inhibition [Chasin & Harris, 1977; Wells & Kramer, 1981]. More selective phosphodiesterase inhibitors have been sought [Weiss & Hait, 1977; Appleman et al., 1982], and some of those which have been synthesized have been shown to potentiate the physiologic effects (e.g.: bovine coronary artery relaxation) of adenyl cyclase and guanyl cyclase activators [Poch & Reich, 1983; Lorenz & Wells, 1983]. A more intriguing use of selective inhibitors has been in the treatment of diseases characterized by cyclic nucleotide metabolism abnormalities.

Cyclic nucleotides mediate numerous physiologic processes, and abnormalities in cyclic nucleotide metabolism are responsible for pathophysiologic processes. Abnormalities in cyclic nucleotide metabolism can occur at any level, with abnormalities in hormones, receptors, nucleotide proteins, catalytic sites, phosphodiesterases, protein kinases, calmodulin or intracellular ions possibly responsible for pathophysiologic processes [Hamet et al., 1984]. Phosphodiesterase inhibitors have been used to treat disease processes characterized by decreased levels of cAMP or cGMP, whether the cyclic nucleotide metabolism abnormality was known to be at the level of cyclic nucleotide synthesis or degradation. Various disease conditions improved by phosphodiesterase inhibition include psoriasis, asthma, certain inflammatory

states, adult onset diabetes, leukemia and other malignancies [Weiss & Hait, 1977]. As the roles of cyclic nucleotides in physiology and pathophysiology become better defined the effective employment of selective phosphodiesterase inhibitors becomes more likely. More selective phosphodiesterase inhibitors might have potential not only in the diseases mentioned above but also in abnormalities of neurotransmission in which the calmodulin-stimulated phosphodiesterase plays a role [Appleman et al., 1982] or in retinal degenerative diseases in which deficient levels of cGMP phosphodiesterase have been implicated [Farber, 1982].

This study was concerned with the effects of a reportedly selective phosphodiesterase inhibitor (HL725, Hoescht-Roussel Pharmaceutical, Inc.) on rabbit IOP and ciliary process cyclic nucleotide production. Rabbit ciliary processes contain a beta-adrenergic receptor-adenyl cyclase complex which is postulated to effect decreases in aqueous humor formation and IOP upon activation by agents which are known to increase ciliary process intracellular cAMP levels [Gregory et al., 1981; Caprioli & Sears, 1984]. It was theorized that a phosphodiesterase inhibitor shown (at a certain concentration range) to be selective for cAMP phosphodiesterase [Ruppert & Weithmann, 1982; Ruppert, 1983] could selectively increase ciliary process cAMP and result in a decreased IOP. The agent (HL725) did cause small decreases in IOP when topically applied and did slightly potentiate the hypotensive effect of isoproterenol when injected intravitreally, but did not decrease IOP when administered intravitreally. Measurements of ciliary process cyclic nucleotide production demonstrated that HL725 increased cAMP and

cGMP levels in the same range of HL725 concentration. Let us examine more closely HL725's effects on IOP and on ciliary process cyclic nucleotide production as well as our knowledge of cyclic nucleotide system in the eye and agents affecting this system.

Adenyl cyclase and beta receptors have been identified in the epithelial membranes of rabbit ciliary processes [Tsukahara & Maezawa, 1978; Bromberg et al., 1980]. Guanyl cyclase activity has been demonstrated in the soluble and particulate fractions of rabbit ciliary processes [Gregory, personal communication], indicating the probable existence of both cytosolic and membrane-associated guanyl cyclase in this tissue.

As early as 1966 Shanta et al. documented the presence of phosphodiesterase in the apical nonpigmented epithelial cells of the ciliary processes. Studies at the time were unable to distinguish between the various forms of phosphodiesterase, so one can only speculate as to the type or types of phosphodiesterase activity which that study showed to be present in various ocular tissue including ciliary processes, conjunctiva, corneal stroma and endothelium, anterior chamber angle endothelium, retinal rod and cone outer and inner segments, outer limiting membrane, outer and inner nuclear layer, outer and inner plexiform layers, and the choroid. One might speculate on the basis of present knowledge [Farber, 1982] that the retinal rod outer segments would contain a calcium-calmodulin-independent cGMP phosphodiesterase and a light-activated cAMP phosphodiesterase [Yamazaki et al., 1985] and the corneal epithelium might contain some forms of cAMP phosphodiesterase [Farber,

1982], and on the basis of the present study that the ciliary processes must contain cAMP and cGMP phosphodiesterases (or possibly the cGMP-stimulated cyclic nucleotide phosphodiesterase). Studies by Gregory and Bausher [personal communication] indicate the presence of phosphodiesterases degrading cAMP and cGMP in soluble and particulate fractions of rabbit ciliary processes. No studies have been done to further characterize and more specifically indentify and localize the rabbit ciliary process phosphodiesterases.

Why did HL725 in one concentration range stimulate both cAMP and cGMP production in the rabbit ciliary processes? HL725 was shown to have potent platelet aggregation inhibitory and hypotensive vasodilatory properties not mediated by alpha- or beta-adrenergic receptors [Lal et al., 1981 & 1984; Ruppert & Weithmann, 1982], and because it had a strong inhibitory effect on cAMP phosphodiesterase in platelets and vascular tissues it was proposed that its physiologic effects were mediated by cAMP phosphodiesterase inhibition [Lal et al., 1981]. Further studies of HL725's effects on platelet cyclic nucleotide phosphodiesterases indicated that it was an effective inhibitor of cAMP and cGMP phosphodiesterase in platelets, with cAMP phosphodiesterase inhibited at concentrations betweeen 10^{-12} and 10^{-8} M and cGMP phosphodiesterase inhibited between 10^{-8} and 10^{-5} M HL725. Platelet cAMP production was stimulated at concentrations between 10^{-7} and 10^{-4} M, and platelet aggregation was inhibited at concentrations between 10^{-13} and 10^{-10} M. [Ruppert & Weithmann, 1982]. An additional investigation further characterized HL725's effects on various platelet phosphodiesterase fractions. HL725 inhibited high affinity cGMP, low affinity

cyclic nucleotide, and high affinity cAMP phosphodiesterases from platelet extract separated by DEAE-Biogel A chromotography (i.e.: soluble fraction phosphodiesterases). cGMP phosphodiesterase (PDE) was inhibited with $K_i = 4 \times 10^{-7}$ M and $IC_{50} = 7 \times 10^{-7}$ M at 0.5 microM cGMP; cyclic nucleotide PDE with $K_i = 4 \times 10^{-7}$ M and $IC_{50} = 6 \times 10^{-10}$ M at 10 microM cGMP; and cAMP PDE with $IC_{50} = 2\text{-}5 \times 10^{-6}$ M at 0.5 microM cAMP (all molarity values represent HL725 concentration unless otherwise specified). The washed particulate fraction also contained PDEs inhibited by HL725: a cGMP PDE with $IC_{50} = 1.5 \times 10^{-7}$ M (at 0.5 microM cGMP) and a cAMP PDE with $IC_{50} = 3\text{-}7 \times 10^{-6}$ M (at 0.5 microM cAMP). Although, as the author reports, there is "a remarkable split between the inhibitory effects of HL725 upon the low K_m (high affinity) cAMP and cGMP phosphodiesterase activities" and the cytosolic and membrane-associated cAMP PDE inhibition may be responsible for HL725's antiaggregatory effects [Ruppert, 1983], it is obvious that HL725 may be affecting cGMP and low affinity cyclic nucleotide phosphodiesterases and exerting some of its effects by those actions as well.

Platelet function and smooth muscle relaxation are regulated by changes not only in cAMP but also in cGMP. It has been known for years that platelet aggregation is inhibited by increases in cAMP, but platelet aggregation recently has been shown to be inhibited by increases in cGMP as well [Hamet et al., 1984; Hidaka & Endo, 1984; Bohme et al., 1984]. Vascular smooth muscle relaxation also is caused by increases in both cAMP and cGMP [Hamet et al., 1984; Hidaka & Endo, 1984; Bohme et al., 1984]. It is apparent therefore that both cAMP and cGMP increases can mediate vascular smooth muscle relaxation and platelet ag-

gregatory inhibition. Platelets, lung tissue, vascular smooth muscle, and rod outer segments all contain a specific cGMP binding protein which has PDE activity; this protein is distinct from calmodulin-sensitive and cGMP-stimulated PDEs [Hamet et al., 1984]. This is only one form of PDE present in platelets and vascular smooth muscle which along with the other PDEs present must be involved in cAMP and cGMP regulation in these tissues. Recent reports confirm that activation of guanyl cyclase brings about smooth muscle relaxation [Ignarro & Kadowitz, 1985; Rapoport et al., 1983]. A cGMP-dependent protein kinase has been located in vascular smooth muscle cell membranes, and smooth muscle relaxation appears to involve removal of calcium from the cytoplasm [Lincoln & Johnson, 1984]. cGMP apparently activates cGMP-dependent protein kinase which then stimulates sarcolemmal calcium extrusion ATPase [Popescu et al., 1985].

HL725's potency in inhibiting platelet aggregation and inducing vascular smooth muscle relaxation may be dependent on its inhibition of cAMP PDEs, cGMP PDEs, or both. The selectivity of phosphodiesterase inhibitors can vary from one tissue to another [Kramer et al., 1977]. The resultant physiologic effect of a phosphodiesterase inhibitor on a tissue will be dependent on the ratio of various forms of PDE in that tissue and the selective effect of the phosphodiesterase inhibitor on that tissue's PDEs. The proportional increases in cAMP and cGMP as well as the dependence of the intracellular message on calcium can affect the cell's physiologic response [Goldberg et al., 1973]. HL725 appears to have a selective effect on cAMP and cGMP phosphodiesterases depending on its concentration in platelets. Its antiaggregatory ef-

fect may be the result of increases in cAMP or cGMP or both. HL725's vasodilatory properties could be dependent on increases of one or both of the cyclic nucleotides.

Although one must consider the limitations of measurements of gross tissue cyclic nucleotide levels at one point in time (changes in cyclic nucleotide concentrations may occur rapidly, measurement in an entire tissue provides only a gross picture of the individual cellular and subcellular levels of cyclic nucleotides, and the physiologic effects may be dependent on the rate and ratio of the changes as well as the involvement of other intracellular messengers), it can be concluded from the measurements of cyclic nucleotide levels that HL725 increases cAMP and cGMP above basal levels in ciliary processes. HL725 is not a selective inhibitor of ciliary process phosphodiesterases. Is the small ocular hypotensive effect of HL725 a result of simultaneous stimulation of both cyclic nucleotides? If cAMP and cGMP have similar influences on AHF, assuming that HL725 is penetrating the ciliary process cells and effecting increases in both cyclic nucleotides one would expect to see significant decreases in IOP from diminished AHF. The question of cellular penetration will be addressed later. For the moment let us assume that HL725 is simultaneously increasing both cyclic nucleotide levels in ciliary processes *in vivo* as it does *in vitro*. What are the possible explanations for it causing only small changes in IOP? Increases in cGMP may be activating cGMP-stimulated cAMP PDE thereby counteracting HL725's increases of cAMP by causing a delayed stimulation of cAMP degradation. The new ratio of cAMP to cGMP brought about by HL725 may result in only minor cAMP-mediated effects (and

therefore small decreases in IOP). Increases in a cGMP may mediate ocular hypertensive effects which are counteractive to the cAMP-mediated ocular hypotensive effects. (Indeed one group of investigators reported that topical application of agents known to stimulate guanyl cyclase resulted in increased aqueous humor cGMP concentrations and IOP [Krupin et al., 1977], although other investigators were unable to confirm these findings [Sears, 1984].) Increases in cGMP may be attributable to vascular smooth muscle cGMP level increases in the ciliary processes, not to cGMP presence in the NPE cell.

HL725 may be affecting the cyclic nucleotide levels in other ocular tissues and thereby changing other components of aqueous humor dynamics which could counteract a reduction in AHF. Cyclic nucleotides serve as intracellular messengers regulating the cellular function of many other ocular tissues besides the ciliary process nonpigmented epithelium. They are involved in the regulation of ocular functions such as retinal visual transduction, corneal chloride pump, blood aqueous barrier stability, ciliary muscle relaxation, and perhaps aqueous outflow [Farber, 1982]. Undoubtedly many other roles for cyclic nucleotides in regulation of ocular tissue function will be discovered. Based only upon the presently available data it is difficult to draw a unifying hypothesis concerning the intracellular messenger system and its control of AHF and IOP. The ciliary process adenyl cyclase-receptor complex has introduced a possible mechanism of AHF control whereby adenyl cyclase stimulants effect decreases in AHF (and IOP). What are the ocular hypo- or hypertensive effects of other agents known to stimulate or inhibit adenyl cyclase in the classic model of the intracel-

lular second messenger system previously outlined? Dopamine can cause ocular hypotension (and this effect can be blocked by haloperidol) [Shannon et al., 1976], but possible involvement of dopamine receptors, which can be stimulatory (D_1) or inhibitory (D_2) of adenyl cyclase, have not been studied in ciliary process membranes. Alpha$_2$-adrenergic agonists lower IOP [van Zwieten & Zimmermans, 1984] and AHF [Lee et al., 1984], and both alpha$_1$ and alpha$_2$ antagonists also lower IOP [Mittag et al, 1985]. In the classic model, alpha$_2$ receptors are inhibitory of adenyl cyclase and would be expected to decrease cAMP and either prevent ocular hypotension or cause hypertension. Beside the studies correlating beta-agonist, cholera toxin, and forskolin stimulation of adenyl cyclase with increases in cAMP and subsequent decreases in AHF, it is difficult to propose a correlation between other agents, stimulatory and inhibitory receptors, effects on cAMP and AHF based upon presently available information. It will be of interest to attempt localization of dopamine, adenosine, muscarinic cholinergic, and prostaglandin receptors coupled to adenyl cyclase in the ciliary processes and to correlate the effects on AHF with stimulation of these receptors (if present).

What of the possible role of cGMP as an intracellular messenger in ciliary process nonpigmented epithelial cells regulating AHF? Little information is available on cGMP stimulation and ocular tension. As previously mentioned, one study indicated that agents stimulating the production of cGMP resulted in ocular hypertension [Krupin et al., 1977], although this finding has not been duplicated [Sears, 1984]. Guanyl cyclase and a cGMP phosphodiesterase do exist in ciliary pro-

cesses [Gregory & Bausher, personal communication], and HL725 causes an increase in ciliary process cGMP. Does cGMP serve as an intracellular messenger in ciliary processes, involved in regulation either of AHF or of some other physiologic process, or is guanyl cyclase a non-functional enzyme present in the ciliary processes only as a remnant of its neuroectodermal layer origin (the layer of the secondary optic vesicle from which the sensory retina with its functional guanyl cyclase developed) [Newell, 1982]? In light of cGMP's stimulation by ascorbate [Murad et al., 1981] and the suggestion of active secretion of ascorbic acid across the ciliary process [Sears, 1980] with ascorbic acid transport negatively correlated with AHF [Sears, 1980; Bar-Ilan, 1984], it is possible that intracellular ascorbate concentrations controlled by an ascorbate transport mechanism might regulate increases in cGMP and a subsequent increase in IOP (or other physiologic effect). Another possibility is that cGMP might act as a monitor of the redox state of the tissue, preventing oxidation or destruction (by free radicals) of cellular components [Murad et al., 1979].

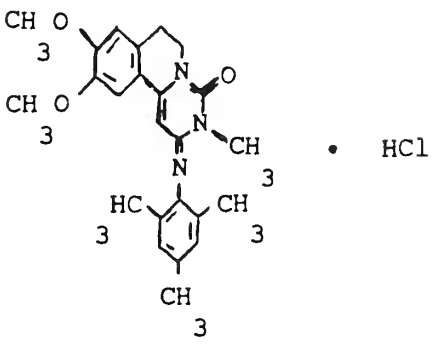
Could calcium also function as an intracellular messenger in the ciliary process nonpigmented epithelium? One of the phosphodiesterases in the ciliary processes could be a calcium-calmodulin-stimulated phosphodiesterase. Vanadate, an agent known to affect not only the sodium/-potassium-ATPase but also the calcium-ATPase [Manalan & Klee, 1984], has been shown to decrease AHF and IOP [Podos et al, 1984; Mittag et al, 1984]. The sodium/potassium-ATPase is known to be present on the lateral interdigitations of NPE cells and to be involved in the secretory mechanism responsible for AHF [Sears, 1980], and the calcium-

ATPase may also be involved. Calcium is responsible for the regulation of so many cellular functions [Rasmussen et al., 1984] that it is probable to have a role in the function of the ciliary process NPE and in AHF.

The model of the second messenger system regulating the NPE cell is far from complete. An adenyl cyclase-receptor complex present in the NPE plasma membrane promotes decreases in AHF and IOP when stimulated by beta-agonists, cholera toxin, and forskolin. In the ciliary processes phosphodiesterases are present, though their types and ratio are not known. Increases in cGMP in the ciliary processes can be stimulated, but its location and role, if any, are not known. A possible role for calcium as an associated intracellular messenger has yet to be defined. It will be exciting in the future to attempt to locate additional (inhibitory as well as stimulatory) adenyl cyclase receptors (by radioligand binding or other methods), to characterize and localize various forms of phosphodiesterase (with monoclonal antibodies [Hurwitz et al., 1984] and enzymatic studies), to localize guanyl cyclase and investigate its possible functional role, and to study the possible activity of calcium within the ciliary processes and individual cell types. As more information is obtained a clearer picture of the intracellular messenger system and its regulation of AHF will be able to be constructed.

To conclude the discussion of HL725's minimal hypotensive effect, one must consider the problems of ocular and cellular penetration or bioavailability of the substance. HL725 or trequensin hydrochloride (9.10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2H-pyrimido[6

,1-a] isoquinolin-4-one hydrochloride):



is a hydrochloride salt of the isoquinoline derivative drawn above. It is relatively insoluble in saline and forms a flocculent precipitate in BSS. The compound's relative insolubility in saline required that suspensions of the substance be employed at high concentrations. To prolong contact of the substance with the cornea and to increase penetration, a dispersion in BSS and hydroxypropyl methylcellulose was prepared. Unfortunately HL725 formed a flocculent precipitate in BSS which may have been a less reactive (and perhaps less potent) salt of the substance. Relatively poor ocular bioavailability and cellular penetration might be cited as a possible explanation for this compound's only slight effectiveness as an ocular hypotensive agent. The agent was effective in vitro in increasing ciliary process cAMP and cGMP levels and therefore must have penetrated the cells sufficiently to cause phosphodiesterase inhibition, although one can only speculate as to what extent or in what concentrations the compound crossed the plasma membrane. The substance did exert slight ocular hypotensive effects when applied topically and did potentiate the effects of topically applied isoproterenol when injected intravitreally, so one could presume that some amount of HL725 reached the site of action in the

ciliary processes when administered topically and intravitreally. Poor ocular bioavailability and cellular penetration may partially account for HL725's minimal effectiveness in mimicking a cAMP-mediated response and in potentiating the effects of an agent known to stimulate adenyl cyclase.

Why did intravitreal injections of HL725 result in no changes in IOP but potentiate the hypotensive effect of isoproterenol? One would expect that any problem with bioavailability with intravitreal injections would be equal in the presence or absence of isoproterenol. HL725 increased cAMP and cGMP levels in the rabbit ciliary processes in vitro. The cyclase activity ratio (ratio of cAMP activity in the presence of agent to the basal cAMP activity) varied between 1.7 and 4.2, with HL725 concentrations between 10^{-7} and 10^{-4} M, whereas the cyclase activity ratio previously reported for isoproterenol was close to 6 [Caprioli & Sears, 1984]. Increases of cAMP resulting in cyclase activity ratios between 2 and 3 should be sufficient to maximally activate protein kinases [Butcher, 1984]. It is possible that the amount of HL725 actually reaching the ciliary process phosphodiesterases when injected intravitrally was insufficient to stimulate cAMP increases two- to three-fold above basal but was sufficient to potentiate the response brought about by low concentrations (submaximal) of isoproterenol.

Early in the course of this study, when HL725 had shown only small ocular hypotensive effects and its effects on ciliary process cyclic nucleotide levels were unknown, it was postulated that HL725 might cause

only small effects because of low basal cAMP levels. If basal turnover of cAMP were low then phosphodiesterase inhibition might result in cAMP stimulation insufficient to cause a significant physiologic (IOP) effect. The IOP circadian rhythm suggested the possibility of a circadian rhythm of ciliary process cAMP levels (possible mediators of AHF) as well. Administration of HL725 at the time of a cAMP level nadir or to a tissue with low basal cAMP turnover would be unlikely to result in dramatic cAMP increases. Submaximal doses of isoproterenol might stimulate the adenyl cyclase system enough to give the phosphodiesterase inhibitor an opportunity to work. HL725 was employed in conjunction with isoproterenol (in a dose shown to be minimally but significantly hypotensive, and therefore capable of providing submaximal stimulation of the cyclase system), and it did potentiate isoproterenol's ocular hypotension, but the delta IOP (treated eye IOP minus control eye IOP) was not remarkably different from that seen with topical HL725 alone. HL725 probably caused small changes in IOP not because of low basal cAMP turnover in the ciliary processes but because it nonselectively affected both cyclic nucleotides or because its bioavailability was too low.

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