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The Ohio State University

Ph.D. 1985

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THE EFFECTS OF AGE ON MUSCARINIC AND
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OF THE RAT URINARY BLADDER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Gregory Allen Ordway, B.S., R.Ph.

The Ohio State University
1985

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ACKNOWLEDGEMENTS

Continuous encouragement from my parents, Dwight and Thelma, has always been my greatest asset.

** **

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"It ... is the greatest sensation of existence: not to trust, but to know."

"But she knew that there was no meaning in motors or factories or trains, that their only meaning was in man's enjoyment of his life, which they served—and that her swelling admiration at the sight of an achievement was for the man from whom it came, for the power and the radiant vision within him which had seen the earth as a place of enjoyment and had known that the work of achieving one's happiness was the purpose, the sanction and the meaning of life."

from ATLAS SHRUGGED, Ayn Rand
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  Neurological disease
  Neuropharmacology of Aging

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CHAPTER I
INTRODUCTION

1.0 Overview

Research on the neuropharmacology of aging has focused heavily on the aging brain, heart and vascular system (Roth and Hess, 1982). In terms of organs controlled by the autonomic nervous system, the heart and vascular system have received the most attention. This dissertation is the first detailed experimental investigation of the effect of age on the autonomic control of the urinary bladder to my knowledge. The studies herein constitute a thorough examination of the effect of age on the responsiveness of the bladder to alpha adrenergic and cholinergic agents.

This introductory chapter first concentrates on the anatomy, physiology and pharmacology of the bladder, with particular emphasis on adrenergic and cholinergic innervation. Thereafter, the physiology and pathology of the aging bladder is discussed. This chapter will provide the reader with an understanding of how a bladder which exhibits an altered responsiveness to autonomic influences may cease to function properly.
1.1 Anatomy, Physiology and Pharmacology of the Urinary Bladder

1.1.1 Anatomy of the urinary bladder.

The urinary bladder is a hollow muscular organ lying immediately dorsal to the symphysis pubis and ventral to the intestinum rectum on the pelvic floor. It receives urine from the kidneys via the ureters, which enter the bladder dorsally just above the bladder outlet. The outlet of the bladder merges with the urethra which provides an opening through which urine can be expelled. The bladder is divided into two general regions: the bladder body and the bladder neck. The bladder body is the largest portion of the bladder and includes the detrusor muscle. The bladder neck is the region beginning at the level of the ureters and extending rostrally to include the outlet and the proximal portion of the urethra (Figure 1). The different regions of the bladder have been assigned multiple names, resulting in a confusing and often misleading anatomical terminology. For clarity of interpretation of the literature, the anatomical names used by authors of respective literature citings will be used in this discussion and can be visualized with respect to the body and neck regions by examining Figure 1.

Histology. The bladder consists of three different types of tissue: epithelium, connective tissue and smooth muscle. The inner luminal layer of the bladder is composed of transitional epithelium that is capable of preventing diffusion of water from blood vessels of the bladder wall to the often hypertonic urine (Bloom and Fawcett, 1975). Beneath this layer is a submucosal layer of connective tissue,
Figure 1. The regions of the urinary bladder.
consisting of collagen and elastin, providing strength and compliance to the bladder wall. External to this submucosal layer are 2 to 3 layers of smooth muscle tissue with connective tissue interspersed throughout. This connective tissue is composed largely of collagen; estimates of bladder collagen content range from 25% (Swaiman and Bradley, 1967) to 57% (Susset et al., 1978) of bladder dry weight, with the bladder body and neck having nearly equal amounts. An important role for collagen in the quality of bladder contraction has been suggested by Susset and Regnier (1981) demonstrating reduced contractions in bladders with greater collagen content.

The proximal portion of the urethra consists of an inner epithelial layer much the same as the bladder, with a submucosal layer rich in elastic fibers just below the epithelium (Goss, 1973); as in the bladder, smooth muscle fibers are also interspersed with collagen and elastin. In humans, the entire urethral length is 18 to 20 cm in the male and 4 cm in the female. Although this dissertation emphasizes the proximal urethra, the importance of the entire length of the urethra in maintaining and regulating continence is not overlooked.

**Smooth muscles of the bladder.** The smooth muscles of the 2 bladder regions are anatomically distinct. The detrusor muscle is found in the bladder body and is the largest muscle of the bladder. The bladder neck region contains smooth muscles in the area of the bladder outlet, the trigone muscle and muscles of the proximal urethra. The detrusor muscle consists of 3 layers of smooth muscle fibers: an inner layer of primarily longitudinal muscle fibers, a middle layer consisting of fibers arranged more or less in a circular manner, and
an outer layer of fibers arranged longitudinally. The inner longitudinal muscle fibers extend down into the urethra and comprise the inner longitudinal muscle layers of the urethra. The fibers of the middle circular layer are very thinly and irregularly scattered in the bladder body and are arranged obliquely. Near the bladder neck region the middle circular layer becomes thickened and more prominent and does not extend down into the urethra (Goss, 1973). The outer longitudinal layer of smooth muscle of the bladder converges and thickens at the bladder neck and inserts into the area where the urethra meets the bladder. A small group of these fibers go on to loop around and form the anterior and lateral portions of the bladder neck (Hutch, 1971).

Another muscle which lies in the bladder neck region, distinctly separate from other muscles of the region, is the trigone muscle (superficial trigone). This muscle is a continuation of ureteral smooth muscle and lies just below the dorsal mucosa of the bladder neck and superficial to the deep trigone (bladder neck longitudinal and circular fibers). This muscle extends down into the urethra and consists of a preponderance of longitudinal muscle fibers (Hutch, 1971). Embryologically, the trigone muscle is derived from mesoderm, as are the ureters from which the trigone originates. However, the bladder body muscle and the other bladder neck muscles, which are in a large part a continuation of the body muscle, are derived from the endodermal cloaca (Lumley et al., 1975).

Another muscle important in bladder function is the external urethral sphincter. These skeletal muscle fibers encircle the smooth
muscle of the urethra and contribute to the retention of urine during urine storage. Muscles of the external urethral sphincter are horseshoe shaped, covering the urethra ventrally and medially from the bladder outlet to the urogenital diaphragm (Donker et al., 1976).

1.1.2 Neurophysiology of the urinary bladder.

Although the urinary bladder is an involuntary organ, conscious control over when urine expulsion occurs is possible because of afferent and efferent nerve connections with the brain. Control is mediated via a complex network of neurons, which can be divided into four general categories based on location: 1) central neurons, 2) spinal neurons with both ascending and descending axons, 3) peripheral autonomic and voluntary motor neurons and 4) sensory neurons. The role of the brain in the control of bladder function is twofold: 1) processing sensory information concerning the fullness of the bladder, and 2) mediating a tonic inhibition of autonomic nuclei located in the spinal cord which control bladder contraction. This network of neurons is diagrammatically represented in Figure 2 and will be discussed in the following paragraphs as a loop of nerves beginning with cortical efferents to the bladder and ending with afferents from the bladder returning to the cortex.

Central input. Central motor regulation of bladder function originates in the 5th layer of the frontal lobe of the cerebral cortex. Here, cell bodies send dendrites out to the 1st layer of the cortex to receive input from sensory axons (Bradley, 1978). Axons from layer V project to the brainstem, passing through the thalamus and basal
Figure 2. Diagram of nerve connections between the brain, spinal column and urinary bladder.

Arrows depict the direction of impulse flow. Proprioceptors are located in the smooth muscle of the bladder and the skeletal muscle of the external urethral sphincter. Abbreviations used are BSDN, brain stem detrusor nucleus; NVP, nucleus ventralis posterolateralis; SAC, sympathetic autonomic cells; DMN, detrusor motor nucleus (parasympathetic); DRG, dorsal root ganglia; PMN, pudendal motor nucleus; HG, hypogastric ganglion; PG, pelvic ganglion.
Figure 2
ganglia, where they innervate cell bodies of the brain stem detrusor nucleus (BSDN) (Bradley and Conway, 1966). The exact pathways which comprise the cortical input to the BSDN are not known; however, stimulation of cortical regions can result in both facilitation and inhibition of detrusor contractions (Gjone and Seteklev, 1963). Cortical neurons which synapse in the BSDN are thought to provide regulation of the reflexive bladder activity (Hald and Bradley, 1982).

The BSDN was originally thought to be the locus coeruleus (Bradley and Conway, 1966), lying in the pontine-mesencephalic gray matter. However, it has since been shown to be rostral to the locus coeruleus, in the nucleus tegmentolateralis dorsalis (Satoh et al., 1978; Loewy et al., 1979). Cell bodies of the BSDN have axons which descend via the reticulospinal tract of the spinal cord and terminate on the cells of the intermediolateral cell column of the sacral spinal cord gray matter. The BSDN also receives afferent inputs originating from stretch receptors in the bladder. Thus, the BSDN is essentially a central relay station for impulses traveling to and from the bladder.

Parasympathetic innervation. The intermediolateral cell column, at sacral levels S2 to S4, is termed the detrusor motor nucleus and consists of cell bodies of preganglionic parasympathetic nerves which send axons, via the pelvic nerve, to the parasympathetic ganglia near the bladder. These ganglia of the rat lie outside the wall of the bladder in connective tissue (Alm and Elmer, 1975), whereas ganglia of the cat, dog, and rabbit lie within the bladder wall (El-Badawi and Schenk, 1966). Cholinergic ganglia in humans have been reported to be both inside (Dixon et al., 1983) and outside (Ek et al., 1977) of the
bladder wall. Cholinergic fibers of postganglionic nerves enter the wall of the bladder and innervate the smooth muscles individually via axon varicosities (Hald and Bradley, 1982). Alm and Elmer (1975) demonstrated the existence of a dense network of acetylcholinesterase positive fibers (interpreted to be cholinergic nerves) in both regions of the bladder, more dense than the adrenergic innervation of either region. Alm and Elmer also suggested that adrenergic nerves in the bladder arise from sacral nerve trunks, travelling in the pelvic parasympathetic nerve, since parasympathetic decentralization produced a complete disappearance of adrenergic nerves in the bladder of the rat. On the other hand, hypogastric denervation had little effect on the adrenergic nerve terminal density. Using these denervation procedures, Alm and Elmer demonstrated that both the hypogastric nerve bundle and pelvic nerve bundle contain both adrenergic and cholinergic fibers. Based on the differential effects of 6-hydroxydopamine and reserpine on long and short adrenergic neurons, these researchers have noted that the adrenergic nerves of the rat bladder body are long adrenergic neurons (having a short preganglionic fiber and a long postganglionic fiber) which are sparsely located. Furthermore, short adrenergic neurons densely innervate the bladder neck. While no adrenergic ganglia were found within the bladder wall, clusters of ganglia are found in the connective tissue surrounding the base of the bladder (Alm and Elmer, 1975). These ganglia may be pelvic ganglia and probably give rise to the short adrenergic neurons of the bladder neck since pelvic denervation results in a major decline in adrenergic innervation. The sparse long adrenergic neurons of the bladder body
may be of hypogastric nerve origin, coming from the inferior mesenteric ganglion (hypogastric ganglion), since hypogastric denervation has a slight effect on detrusor adrenergic denervation (Alm and Elmer, 1975). Based on their studies, Alm and Elmer (1975) conclude that the pelvic nerve provides the greatest contribution of both adrenergic and cholinergic nerves to the bladder.

Alm and Elmer (1975) and de Groat (1980) also demonstrated the presence of small intensely fluorescent (SIF) cells in the muscular layers of the bladder wall. These nerves contain norepinephrine as their transmitter and have been proposed to provide an efferent link from the hypogastric ganglion to the pelvic ganglion. It is thought that this link provides sympathetic nerves with regulatory influence over the activity of parasympathetic nerves.

Stimulation of the pelvic nerve results in contraction of both the bladder body (Vanov, 1965; Sibley, 1984) and neck (Slack et al., 1982). By examining the effects of atropine on nerve-stimulated contractions, both Sibley (1984) and Slack (1982) concluded that the major excitatory pathway to the bladder regions is cholinergic. Contractions of the neck following nerve stimulation were only slightly affected by phentolamine, while those of the body were not affected. The possible existence of other non-adrenergic non-cholinergic transmitters and their contribution to bladder contraction following pelvic nerve stimulation will be discussed later in this section.

Knowledge of the complexity of the parasympathetic pelvic ganglia comes from research on feline bladder ganglia. These ganglia differ in location from those of the rat bladder, the implication of which,
if any, is difficult. The parasympathetic ganglia are more complicated than the sympathetic ganglia in that they are innervated by both adrenergic and cholinergic neurons (Figure 3), receiving pre-ganglionic parasympathetic nerve input (cholinergic) from sacral spinal levels S-2 to S-4, and postganglionic sympathetic input (adrenergic) via the hypogastric plexus from lumbar spinal levels (de Groat and Booth, 1980). Adrenergic fibers traveling via the hypogastric nerve may not directly innervate smooth muscle cells, but may only modulate pelvic ganglionic activity. Furthermore, the pelvic ganglia contain at least 3 types of ganglion cells: cholinergic, adrenergic and purinergic. Although the demonstration of these cells has been in feline pelvic ganglia, it is likely that the pelvic ganglion of the rat contains at least cholinergic and adrenergic cells since a loss of adrenergic and cholinergic nerves occurs after parasympathetic denervation (Alm and Elmer, 1975).

Based on electrophysiological studies on the feline pelvic ganglia, de Groat and Booth (1980) postulate that the pelvic ganglia provide the major excitatory input to the bladder, acting as "high pass filters" that amplify parasympathetic excitatory input during micturition. During low stimulus frequencies, that is, while pelvic ganglia are receiving few signals from spinal preganglionic cell bodies, pelvic ganglia are inefficient in transmission. However, when stimulus frequencies are high, these ganglia amplify the parasympathetic input from the preganglionic nerves resulting in enhanced release of neurotransmitter from postganglionic nerve terminals in the bladder. These ganglia are also influenced by both inhibitory and excitatory inputs
Figure 3. Innervation of the pelvic ganglia, hypogastric ganglia and the bladder body and neck.

Muscarinic receptors are depicted as triangles, and adrenergic receptors are depicted as rectangles. Parenthetic alpha and beta adrenoceptors denote less important adrenergic receptors for that region.
Figure 3
from hypogastric nerves, either directly or indirectly, via SIF cells. The role of these sympathetic inputs is not well understood and is believed to be regulatory in nature.

**Sympathetic innervation.** The hypogastric nerve contains axons whose cell bodies are located in the intermediolateral cell column of the spinal cord at T-11 to L-2, comprising the sympathetic nerve trunk innervating the bladder. Cell bodies in this column have axons which terminate on ganglia located in the inferior mesenteric plexus (hypogastric ganglion). Adrenergic ganglia lie outside the bladder wall in man (Benson et al., 1979; Dixon et al., 1983), the rabbit and the rat (El-Badawi and Schenk, 1966; Alm and Elmer, 1975), clusters of which are found in the connective tissue surrounding the bladder base. From the hypogastric ganglion, axons of the postganglionic sympathetic nerves travel into the muscle layer to terminate on smooth muscle cells of the bladder body and bladder vasculature. The adrenergic nerves of the bladder base are likely to be primarily of pelvic nerve origin (as previously discussed); however, some postganglionic sympathetic fibers are believed to innervate the bladder neck (de Groat and Booth, 1980; Tanagho, 1979). Adrenergic innervation of the bladder body, whether of sympathetic or parasympathetic origin, is sparse at the caudal end, becoming increasingly more dense towards the rostral portion. In the bladder neck, a rich supply of adrenergic nerve terminals exists in all species known (El-Badawi and Schenk, 1966; Alm and Elmer, 1975). This varying density of adrenergic innervation throughout the bladder is in contrast to the near continuous cholinergic innervation in both the body and neck (Alm and Elmer, 1975).
Hypogastric nerve stimulation has variable effects on the bladder body and neck. In dogs, hypogastric stimulation results in very small changes in bladder pressure, but causes contraction followed by relaxation of the bladder neck (Tanagho, 1979). Alpha adrenoceptor blockade with phentolamine results in bladder neck relaxation following hypogastric nerve stimulation, while beta receptor blockade with propranolol results in contraction (Tanagho, 1979). The presence of alpha and beta adrenoceptors mediating contraction and relaxation, respectively, in this tissue will be discussed in Section 1.1.3.

Hence, a clear-cut separation of autonomic innervation of the urinary bladder into sympathetic adrenergic fibers and parasympathetic cholinergic fibers does not exist in the rat or cat or probably man. The bladder receives neural input from both the parasympathetic pelvic nerve, containing both cholinergic and adrenergic postganglionic fibers, and the sympathetic hypogastric, nerve containing primarily adrenergic nerves. Cholinergic nerve terminals are of the greatest density in both the bladder body and neck. Adrenergic terminals are sparsely located in the bladder body and densely located in the bladder neck. The origin of these adrenergic neurons in both regions is unclear. Postganglionic sympathetic adrenergic fibers also innervate the pelvic parasympathetic ganglia either directly or via SIF cells. Hypogastric nerve stimulation has little effect on the bladder body and variable effects on the bladder neck. Pelvic nerve stimulation contracts both the bladder body and neck.

**Non-adrenergic non-cholinergic nerves.** A number of non-adrenergic non-cholinergic neurons are known to innervate the bladder. Using
fluorescent histochemical techniques, nerves containing substance P (Alm et al., 1978), vasoactive intestinal polypeptide (Alm et al., 1977), somatostatin, enkephalin (Hokfelt et al., 1978), and ATP (Burnstock et al., 1978) have been demonstrated in the bladder. A role for these substances in the regulation of bladder function is a controversial issue (Elmer, 1978; Andersson and Sjorgen, 1982). Contraction of the bladder following either pelvic or hypogastric nerve stimulation is antagonized only 60% by atropine and not at all by phentolamine (Alm and Elmer, 1975). The degree to which atropine is able to antagonize nerve-stimulated bladder contractions varies from species to species; the guinea pig bladder having the greatest insensitivity (Chesher, 1970), and the human bladder demonstrating no non-cholinergic non-adrenergic component (Sibley, 1984). A popular candidate for the mediator of the non-adrenergic non-cholinergic mechanism is ATP, being liberated from putative purinergic nerves (Downie et al., 1976). However, it has been proposed that the atropine-insensitive portion of bladder contraction is due to the interaction of acetylcholine with junctional receptors unavailable to atropine and very close in proximity to the cholinergic nerve terminal (Ursillo and Clark, 1956). An explanation for interspecies differences in the atropine-insensitive contractions remains to be determined. Further studies in this area of bladder pharmacology are needed in order to determine the importance of non-adrenergic non-cholinergic neurotransmitters or neuromodulators.

The external urethral sphincter. Somatic nerves innervating the periurethral skeletal muscle (external urethral sphincter) have their
origin in the pudendal motor nucleus of the sacral spinal cord. These nerve cell bodies receive input from nerves originating in the motor cortex (Hald and Bradley, 1982). The external urethral sphincter contributes to, but is not a strict requirement for urine storage; even with the loss of these muscles, either surgically (Turner-Warwick, 1968) or by pudendal nerve block (Lapides et al., 1957), the smooth muscle of the urethra is able to maintain closure and storage of urine.

Sensory nerves. Thus far, the nerve input to the bladder has been described. The urinary bladder also has a rich supply of sensory nerves. Sensory nerves originating in the bladder wall transmit information about bladder filling to the brain (supraspinal pathway). Sensory nerves also comprise the afferent link of spinal reflex bladder activity (segmental pathway). The segmental pathway consists of afferent dendrites originating at tension and length receptors in the bladder and pelvic floor musculature. Sensory nerves with dendrites which originate in the bladder muscle follow the pelvic nerve (pelvic sensory nerve) and end in the dorsal root ganglia of the spinal cord. Axons of the dorsal root ganglia synapse locally onto autonomic ganglia (Perkash, 1982) and also travel rostrally to terminate on the brain stem detrusor nucleus (Hald and Bradley, 1982). From here tracts extend to the 1st layer of the cerebral cortex, the origin of the descending motor control of the bladder, thus finishing a loop of nerves carrying information to and from the bladder (Bradley 1978). Also, sensory nerves with dendrites originating in pelvic skeletal musculature follow the pudendal nerve (pudendal sensory nerve) to
their cell bodies in the dorsal gray. Here, axons synapse locally on pudendal motor nerves. Axons also ascend via the dorsal columns and synapse on the nucleus ventralis posterolateralis, which sends axons to the sensorimotor cortex.

1.1.3 Neuropharmacology of the urinary bladder.

**Neurotransmitter and neurotransmitter receptors.** The neurotransmitter released by preganglionic parasympathetic and preganglionic sympathetic nerves is acetylcholine, which activates primarily nicotinic receptors on ganglionic neurons, although inhibitory and excitatory muscarinic receptors are also known to exist. Activation of nicotinic receptors by acetylcholine released from the preganglionic nerves results in firing of the postganglionic nerve (de Groat and Booth, 1980). Alpha and beta adrenoceptors are also thought to exist on parasympathetic ganglia. These receptors are activated by norepinephrine released from terminals of the hypogastric nerve or SIF cells. Thus, the sympathetic adrenergic nerves modulate the activity of parasympathetic ganglia either directly or via norepinephrine containing SIF fibers (de Groat and Booth, 1980).

**Cholinergic muscarinic receptors.** The postganglionic cholinergic neurons send axons to the smooth muscle cells of both the bladder body and bladder neck regions. Terminals of these axons release acetylcholine when the nerve is depolarized. Acetylcholine diffuses across the synapse of the smooth muscle-nerve terminal junction and activates muscarinic receptors. These receptors have been identified by both functional (Vanov 1965; Neghard, 1975; Ek et al., 1977; Levin et al.,
1980) and receptor binding techniques (Levin et al., 1980; Johns, 1983; Nilvebrant and Sparf, 1983). Using receptor binding techniques, the density of muscarinic receptors in the bladder body has been shown to be greater in the bladder body than the neck (Levin et al., 1980; Johns, 1983). Activation of the muscarinic receptor by acetylcholine is followed by a smooth muscle contractile response in both bladder regions (Andersson and Sjorgen, 1982). However, it has been demonstrated in the rabbit bladder that the intensity of response varies among the regions of the bladder. For example, both longitudinal and circular muscles of the bladder body contract to an equal degree following muscarinic receptor activation. However, moving distally from the bladder body, the circular muscles respond less and less to acetylcholine compared to the longitudinal muscles of the same region. Finally, in the urethra, little or no contraction of circular muscles is elicited by acetylcholine, while the longitudinal muscles of the urethra contract (Khanna et al., 1981; Hassouna et al., 1983). These regionally selective responses of bladder smooth musculature most likely occur because of specificity of muscarinic receptor location. However, to date this has not been demonstrated, probably because of difficulty in surgically separating circular and longitudinal muscles.

Several biochemical events have been proposed to occur that connect muscarinic receptor activation by acetylcholine to smooth muscle contraction. Studies involved in the biochemistry of receptor activation have used non-bladder smooth muscle tissue, such as intestinal and vascular tissues and neuroblastoma cell cultures (see McKinney and Richelson, 1984 for review). For the purpose of this dissertation,
the scheme outlined in Figure 4 depicts the current theory of these biochemical events. Occupation of the muscarinic receptor by the agonist promotes the hydrolysis of inositol-containing phospholipids (PIP) by phospholipase C. Thus, phosphatidylinositol 4,5-phosphate is hydrolyzed to 1,2 diacylglycerol and myo-inositol 1,4,5-triphosphate (IP3). IP3 is currently postulated to be the mediator of intracellular calcium mobilization (Joseph, 1984). Another possible metabolite of PIP is phosphatidic acid, which has been proposed to act as a calcium ionophore, allowing the movement of calcium into the cell from extracellular calcium stores (Salmon and Honeyman, 1980; Putney et al., 1984). Whether phosphatidic acid or IP3 mediate the increase in intracellular calcium concentrations may depend on whether agonist induced contraction of smooth muscle is dependent on extracellular or intracellular calcium. The rise in intracellular calcium concentration, from about $10^{-7}$ M to $10^{-5}$ M, results in the binding of calcium to calmodulin, an intracellular calcium binding protein. The calcium-calmodulin complex then interacts with myosin light chain kinase (MLCK) to form an active complex of the kinase. Once activated, MLCK phosphorylates the 20,000 dalton light chains of myosin which allows the activation of myosin Mg$^{2+}$-ATPase activity by actin (formation of active actomyosin) causing the formation of the actin-myosin cross-bridge. ATP hydrolysis by this ATPase provides the energy necessary for crossbridge cycling and the resultant contraction of the muscle, which continues as long as the intracellular calcium concentration is elevated. When the calcium concentration declines, MLCK is inactivated by a phosphatase which dephosphorylates myosin, resulting in
Figure 4. Current hypothesis of the cascade of biochemical events that follow activation of muscarinic and alpha₁ adrenergic receptors.

The activation of alpha₁ adrenoceptors and muscarinic receptors is likely to involve stimulation of different phospholipase C enzymes and not the same enzyme as depicted for the sake of simplicity in the figure. Furthermore, this enzyme is likely to be a phospholipase C which is specific for PIP2 and not for other phosphoinositides. Phosphatidic acid is formed from further metabolism of 1,2-diacylglycerol and may act as a calcium ionophore. Regardless of whether IP3 or phosphatidic acid cause the increase in cytosolic calcium, the end-result is the activation of myosin ATPase by actin and the generation of energy needed for crossbridge cycling (contraction). Abbreviations are as follows: α₁, alpha₁ adrenoceptor; M, muscarinic receptor; PIP2, phosphatidylinositol-4,5-phosphate; IP3, myo-inositol-1,4,5-triphosphate; MLCKᵢ, inactive myosin light chain kinase; MLCKₐ, active MLCK; P, phosphate.
Figure 4

- **PIP2**
- **1,2-diacylglycerol**
- **PI3 phosphatase**
- **IP3**
- **IP2**
- **calmodulin**
- **endoplasmic reticulum**
- **myosin**
- **myosin-P**
- **actin**
- **MLCK**
- **Ca^{2+}**
- **ADP+P**
- **ATP**
- **MLCKa**
- **MLCK1**
- **Ca^{2+}**
relaxation (Squire, 1983; Hartshorne and Persechini, 1984). Muscarinic receptors also stimulate the formation of cyclic guanosine 3',5'-monophosphate (cGMP) in certain tissues, although a role of cGMP in the contraction process is unknown (Exton, 1982). Thus, activation of muscarinic receptors results in a cascade of biochemical events which culminate in the contraction of the muscle; and the exact events which occur are still very much the topic of controversy (Squire, 1983; Stephens, 1984).

Adrenergic receptors. Adrenergic nerves also innervate both regions of the bladder, however, as mentioned previously, to a greater extent in the bladder neck. Norepinephrine, released from adrenergic nerve terminals presumably of both pelvic and hypogastric nerve origin, diffuses across the neuromuscular junction and activates alpha and beta adrenergic receptors located on the smooth muscle cells of both regions of the bladder. The response of the bladder to adrenergic agonists is regionally dependent. Epinephrine, which acts at both alpha and beta adrenoceptors, induces relaxation in the bladder body and contraction in the bladder neck (Levin and Wein, 1979). However, in the presence of propranolol, the relatively non-selective adrenergic agonist norepinephrine produces a concentration dependent contraction of the bladder body. Conversely, the bladder neck relaxes when stimulated by norepinephrine in the presence of phentolamine (Levin et al., 1980). Hence, the bladder body and neck contain both alpha and beta adrenoceptors. Levin and Wein (1979) have demonstrated that the response of either region to a non-selective adrenergic agonist depends upon the relative number of alpha and beta adrenoceptors.
Using receptor binding techniques, they demonstrated that the ratio of alpha to beta adrenoceptors in the bladder neck is approximately 2 while that of the bladder body is about 0.2. Hence, the physiological role of alpha adrenoceptors in the bladder body and beta adrenoceptors in the bladder neck may be of little significance in the normal bladder.

The magnitude of adrenergic responsiveness of circular and longitudinal muscles is regionally dependent. Norepinephrine, presumably acting at alpha adrenoceptors, contracts circular and longitudinal muscle strips of the rabbit trigonal region to an equal degree. Furthermore, in the urethra, norepinephrine induces greater contractions of circular muscles than of longitudinal muscles (Khanna et al., 1981). Isoproterenol, acting at beta adrenoceptors, produces a greater relaxation of circular muscles than of longitudinal muscles of the feline urethra (Hassouna et al., 1983). In the bladder body, norepinephrine produces equal relaxation of longitudinal and circular muscles (Khanna et al., 1981). In summary, beta adrenoceptors mediate relaxation of the bladder body when activated by norepinephrine, and the relaxation occurs in both circular and longitudinal muscles. Beta adrenoceptors may also mediate relaxation of the bladder neck which largely occurs in circular muscles. Alpha adrenoceptors mediate contraction of the bladder neck region, which is more pronounced in circular muscles.

**Alpha adrenoceptors.** Several authors have attempted to classify the alpha adrenoceptors of the urinary bladder. Ruffolo et al. (1980, 1981) classified the receptors of the urinary bladder of the
rat as alpha₁, although the possibility of the existence of alpha₂ adrenoceptors in the bladder was not excluded. The specific region of the bladder used in these studies was not mentioned but is presumed to be primarily bladder body. Barker et al. (1977) and Downie et al. (1975) have demonstrated differences between the alpha adrenoceptors of the rabbit bladder body and the bladder neck. Downie et al. (1975) demonstrated a fourfold to fivefold higher affinity of norepinephrine in the neck than in the body. Furthermore, Barker et al. (1977) reported that the isomeric activity ratios of norepinephrine were significantly different in the two bladder regions. Ueda et al. (1984), using selective alpha₁ and alpha₂ agonists and antagonists, showed that the rabbit bladder body alpha adrenoceptors are of the alpha₁ subtype, while both alpha₁ and alpha₂ adrenoceptors exist in the bladder neck.

The biochemical events which follow alpha₁ adrenoceptor activation are a subject of current controversy (Lefkowitz and Hoffman, 1980; Exton, 1982). Occupation of the receptor is followed by a rise in the cytosolic calcium concentration. Current theory suggests that the rise in intracellular calcium is a result of a receptor-mediated increase in phosphatidylinositol turnover, much the same as seen for activation of muscarinic receptors. Hence, a cascade of biochemical events following alpha₁ receptor occupation by an agonist occur in a manner similar to that which occurs following muscarinic receptor occupation (Figure 4). Alpha₁ adrenoceptors also elevate cGMP in certain tissues, although a role for cGMP in the contraction process is questionable (Exton, 1982).
Beta adrenoceptors. For review of bladder beta adrenoceptors the reader is referred to Ganguly and Vedasiromoni (1976), Larsen (1979) and Andersson and Sjorgen (1982). Briefly, the beta adrenoceptors of the rabbit and pig appear to be of the beta_2_ subtype, while the beta adrenoceptors of the human bladder do not fit into either the beta_1_ or beta_2_ category. As mentioned previously, the beta adrenoceptors are the predominate adrenergic receptors of the bladder body region, although they also exist in the bladder neck. These receptors mediate relaxation in both bladder regions via a receptor linked activation of adenylate cyclase. Increased levels of adenosine 3',5'-monophosphate (cAMP) activate the cAMP-dependent protein kinase which phosphorylates MLCK resulting in the loss of affinity of MLCK for Ca^{2+}-calmodulin. This results in a reduction of the phosphorylated form of myosin and hence, relaxation (Squire, 1983).

1.1.4 Summary of the anatomy, physiology and pharmacology of the urinary bladder in terms of bladder function.

Storage and expulsion of urine are functions shared by the coordinated activities of the bladder body and neck. Much of the knowledge of these functions comes from studies on the cat and rat; the actual process in humans may be somewhat different although more similarities than differences can be cited. During urine storage (filling phase), the detrusor muscle relaxes to accommodate the urine entering the bladder via the ureters. Detrusor relaxation is thought to occur following activation of beta adrenoceptors by norepinephrine released from sympathetic adrenergic neurons (Khanna et al., 1981).
Furthermore, the circular muscles of the bladder neck contract during the filling phase, opposing the leakage of urine by a sphincter-like action. This contraction is likely to be a result of activation of bladder neck alpha adrenoceptors following the release of norepinephrine from adrenergic nerves. As the bladder fills, the intravesical pressure increases only slightly until capacity (400 to 500 ml in humans) is reached. Even before capacity is reached, at about a 100 to 200 ml bladder volume (Tanagho, 1984), stretch receptors of the bladder wall are activated, causing action potentials to run along sensory nerves to the spinal cord. Here, impulses are transmitted to local autonomic nuclei and also sent along axons which carry the information to the BSDN. At this point, reflexive contraction is not initiated because of the activity of descending inhibitory nerves from the brain. The resting bladder pressure during filling is 10 to 15 cm of water. As bladder volume exceeds 350 ml (normal bladder capacity in humans is 400 to 500 ml), there is a sharp increase in bladder pressure to 40 to 100 cm of water. At this time, a bladder contraction ensues as the activity of the reflex afferents overcomes central inhibition. However, bladder contraction and expulsion of urine can be executed at will prior to capacity. This is possible by voluntarily removing inhibition of reflex afferent firing in the BSDN and autonomic nuclei. It appears also possible to voluntarily facilitate reflex afferent input into these nuclei to induce a bladder contraction and expulsion of urine long before bladder capacity is reached (Tanagho, 1984).
During bladder filling, the autonomic nuclei of the sacral spinal cord are sending low frequency impulses to the pelvic ganglia. These ganglia, acting as "high pass filters", are inefficient in transmitting the information to postganglionic neurons which directly innervate the bladder (de Groat and Booth, 1980). The ganglion cells apparently are not completely quiescent since constant tone of circular bladder neck muscles is necessary for preventing loss of urine, although this tone may be mediated through sympathetic adrenergic and cholinergic fibers.

When reflexive activity of afferents overcomes central inhibition, or when central inhibition ceases, or when centrally-mediated facilitation occurs, the preganglionic parasympathetic neuronal firing increases to a maximal rate, which is maintained until voiding ceases (de Groat and Ryall, 1969). The release of acetylcholine from preganglionic parasympathetic nerves results in activation of nicotinic receptors on the pelvic ganglion cells. These ganglion cells, also influenced by the activity of SIF cells and sympathetic adrenergic neurons, amplify signals of the preganglionic nerve when the rate of firing of that nerve exceeds a critical frequency (de Groat and Saum, 1976). The result is an increase in firing of the postganglionic nerve and release of acetylcholine which activates excitatory muscarinic receptors of the bladder body and neck. Contraction of the trigone muscle exerts tension on the ureterovesicular junction resulting in ureteral occlusion, preventing vesicoureteral reflux (Tanagho, 1984). Muscarinic receptor mediated contraction of both longitudinal and circular muscles of the bladder body sharply raises intravesicular
pressure. Furthermore, contraction of longitudinal muscles of the neck shortens the neck region (shortening the proximal urethra) which draws open the outlet and allows the expulsion of urine. Beta adrenoceptor mediated relaxation of the urethra may also contribute to the decline in urethral resistance during voiding (Hassouna et al., 1983).

The contraction of the detrusor and neck muscles is maintained until all urine is expelled at which time the detrusor and longitudinal neck muscles relax. Circular neck muscles resume their tone and bladder filling can begin again. One can exert voluntary cessation of urine flow during the micturition process by contracting the periurethral skeletal muscles which produces an intraurethral pressure greater than the bladder pressure (Kleeman, 1970).

Since the act of voiding requires the existence of possibly several neurotransmitters which act at several types of neuroreceptors, it is not surprising that many drugs can interfere with micturition (see Table 1; Wein, 1982). Thus, drugs can be both detrimental to proper bladder function and also used pharmacologically to correct bladder dysfunction. The role of drugs in the treatment of bladder dysfunction will be discussed in Section 1.2.1.

1.2 Aging of the Urinary Bladder and Incontinence in the Elderly

The urinary bladder is an organ often given little consideration by the young and adult alike. However, as one becomes older, reaching and exceeding 60 years of age, the importance of the function of the bladder becomes less and less an oversight. This increase in awareness of an organ once overlooked may be attributed to a decline in the
Table 1

Acknowledged and Potential Effects of Various Pharmacologic Agents on Urine Storage and Emptying

<table>
<thead>
<tr>
<th>Agent</th>
<th>Facilitate Emptying</th>
<th>Facilitate Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increase Bladder Contractility</td>
<td>Decrease Outlet Resistance</td>
</tr>
<tr>
<td>Acetylcholine-like agents</td>
<td>++†</td>
<td></td>
</tr>
<tr>
<td>Anticholinesterases</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Digitalis</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>α-Adrenergic blockers</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>β-Adrenergic stimulators</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Centrally acting muscle relaxants†</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Polysynaptic inhibitors</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Directly acting skeletal muscle relaxants</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Atropine-like agents</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Ganglionic blockers</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Musculotropic blockers</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Antihistamines</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Phenothiazines</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Narcotics†</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tricyclic antidepressants</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-Adrenergic stimulators</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>L-Dopa</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Amphetamines</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>β-Adrenergic blockers</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*++—accepted, widely acknowledged clinical effects, +—theoretical or laboratory effects and/or effects that are not widely clinically acknowledged.
†These agents act primarily through central effects and may cause decreased emptying on the basis of oversedation or bladder inhibition.

—Reproduced from Wein, 1982.
once 'normal' function of the bladder, often made apparent by an increase in the number of trips to the restroom. If it occurs that the aged person cannot reach the restroom in time to urinate, then the condition is termed geriatric urinary incontinence. If continence is not restored by surgical, psychological or pharmacological means, then the elderly individual risks depression due to the feeling of 'loss of control', social outcasting, and/or financial burden from medical bills or nursing home costs. (Ouslander et al., 1982; Griffin, 1983). Hence, urinary incontinence in the elderly is a source pathological, psychological, social and/or economic difficulties. The importance of an understanding of how aging effects bladder function is necessary for further progress in the treatment of bladder dysfunction in the elderly. The objective of this section is to briefly examine the incidence, classifications, and causes of incontinence and to review literature that examines the effects of age on bladder function.

1.2.1 Urinary incontinence.

Urinary incontinence, the uncontrolled loss of urine, is a symptom of an underlying pathology that adversely effects bladder function either directly or indirectly. Urinary incontinence can be caused by many different diseases including those in areas responsible for the control of the bladder, e.g. Parkinsonism, Alzheimer's disease, multiple sclerosis, diabetes mellitus (Williams and Pannill, 1982; Griffin, 1983) and diseases of the bladder itself, e.g. bladder cancer, bladder diverticulum, urethral closure secondary to prostatic hypertrophy
(Jaffe, 1978). Furthermore, trauma to nerves innervating the bladder at spinal (Bradley and Andersen, 1976) and supraspinal levels (Tara-bulcy, 1974) can also result in incontinence. Many diseases resulting in incontinence are more prevalent in elderly patients (Parkinsonism, Alzheimer's, prostatic hypertrophy, bladder cancer), and, hence, many studies examining geriatric incontinence do not present a clear picture as to what effects age alone has on bladder function. In fact, very few studies exist that define changes that occur in the aged bladder without the complication of disease. These important studies will be critically examined after a brief review of the incidence, classification, cause and treatment of geriatric urinary incontinence.

Incidence of urinary incontinence. Urinary incontinence effects a wide range of ages and is relatively impartial to sex. Continence is generally achieved by the age of 2 years, however some suggest that complete cortical control of the urinary bladder does not occur until the age of 14 (Lapides and Costello, 1969). Incontinence occurs less frequently in young and adult life, with an incidence of 2% of the general population (Hald and Bradley, 1982). However, at ages greater than 65 years, estimates of the incidence of incontinence range from 10 to 20% of those living in the community, and 40 to 50% of elderly persons in nursing homes (Jewett et al., 1981; Ouslander et al., 1982). In fact, incontinence is second only to dementia as the most cited cause for admission of elderly people to nursing homes. Elderly males are reported to have a lower incidence of incontinence than females; about 4 out of 10 elderly individuals suffering from incontinence are male (Griffin, 1983). Surveys on geriatric incontinence
include all types of incontinence, of all causes. In these studies, age has been shown not to be a factor in the prevalence of incontinence (Milne, 1976). That is, within the geriatric population, there seems to be no age-related further increase in the incidence of incontinence. However, one cannot deny that the 10 to 20% incidence in the elderly far exceeds the 2% incidence in the general population. More extensive surveys on the population as a whole, with regard to age, disease states, and types of incontinence are needed before definite conclusions can be made regarding a correlation between age and the likelihood of incontinence.

Classifications of incontinence. Several different types of urinary incontinence exist, and multiple classifications have been proposed according to symptomology, cystometrographic analysis (bladder pressure recordings), and cause (Williams and Pannill, 1982; Griffin, 1983). Willington (1976), Brocklehurst (1979) and Williams and Pannill (1982) review types and classifications of geriatric incontinence. Williams and Pannill conveniently categorize geriatric incontinence into 5 separate categories based on cause and cystometric findings (Table 2). These authors estimate that detrusor instability, also referred to as detrusor hyperreflexia and unstable detrusor contractility, represents the most common type of geriatric incontinence, affecting some 70% of incontinent geriatric patients. In support of this high incidence of detrusor hyperreflexia in the elderly is a study by Overstall et al. (1980) which determined that 57% of 309 geriatric incontinence cases were caused by unstable detrusor. When incontinence secondary to vaginitis, acute infections, drugs, etc. is
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Mechanism</th>
<th>Cause</th>
<th>Usual Cystometric Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detrusor instability</td>
<td>Uninhibited detrusor contractions sufficient to</td>
<td>Defects in central nervous system inhibition; hyperexcitability of afferent pathways; deconditioned micturition reflexes</td>
<td>Involuntary detrusor contractions that cannot be suppressed; spontaneous detrusor contractions greater than 15 cm H₂O at capacity</td>
</tr>
<tr>
<td></td>
<td>cause incontinence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overflow incontinence</td>
<td>Intravesicular pressure cannot exceed intraurethral</td>
<td>Bladder outlet obstruction; detrusor inadequacy; impaired afferent sensation</td>
<td>Little or no detrusor contractions despite high (400 mL) bladder volume; little increase in pressure with high bladder volume; frequently little or no urge to void; high (100 mL) residual volume</td>
</tr>
<tr>
<td></td>
<td>pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphincter insufficiency</td>
<td>Intraurethral pressure involuntarily falls below</td>
<td>Inadequate estrogen to maintain urethral mucosa in women; weakness of pelvic and urethral muscles; urologic surgery; severe neuropathy; urinary infections</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>intravesicular pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional incontinence</td>
<td>Inability to reach toilet in time</td>
<td>Psychological factors; impaired mobility; inconvenient facilities, inflexible staff schedules</td>
<td>Normal</td>
</tr>
<tr>
<td>Iatrogenic incontinence</td>
<td>Multiple mechanisms including loss of awareness to</td>
<td>Various drugs, especially diuretics, sedatives, and autonomic nervous system agents; physical restraints</td>
<td>Varies with cause; all of above can be seen</td>
</tr>
<tr>
<td></td>
<td>bladder cues; decreased bladder or sphincter tone;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>inability to reach toilet in time</td>
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</tbody>
</table>

Reproduced from Williams and Pannill (1978).
disregarded, this figure translates to 72% of geriatric incontinence cases being due to unstable detrusor contractions. It can be concluded from Table 2 that the cause of geriatric incontinence is most often of neurological origin.

**Pharmacological treatment of incontinence.** Treatment of incontinence depends on the type of incontinence and is often based on pharmacological rationale. Williams and Pannill (1982) offer treatment recommendations depending on the condition, and these schedules appear in Table 3. The pharmacological rationale for each of the drug therapies is reviewed thoroughly by Malone-Lee (1982) and will be discussed below; treatment other than drug therapy (surgical, psychological, etc.) is beyond the scope of this dissertation and will not be discussed.

1) Imipramine. Imipramine is a tricyclic antidepressant with anticholinergic and alpha and beta adrenoceptor agonist properties. All these properties may contribute to the effectiveness of imipramine in the treatment of detrusor hyperreflexia: blocking the action of acetylcholine in the detrusor and thereby antagonizing contractions; activating beta adrenoceptors of the detrusor to facilitate filling; and activating alpha adrenoceptors of the urethra to aid in resistance to leakage.

2) Flavoxate. Flavoxate is thought to selectively act on a smooth muscle receptor producing muscle relaxation without antagonizing muscarinic receptors and thereby calms detrusor hyperreflexia.
TABLE 3

Current Treatment Recommendations for Urinary Incontinence

<table>
<thead>
<tr>
<th>Condition</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detrusor instability</strong></td>
<td>Imipramine</td>
</tr>
<tr>
<td></td>
<td>Flavoxate</td>
</tr>
<tr>
<td></td>
<td>Oxybutinin</td>
</tr>
<tr>
<td></td>
<td>Propantheline</td>
</tr>
<tr>
<td><strong>Overflow incontinence</strong></td>
<td>Surgical relief</td>
</tr>
<tr>
<td></td>
<td>Self catheterization</td>
</tr>
<tr>
<td></td>
<td>Bethanechol</td>
</tr>
<tr>
<td></td>
<td>Phenoxybenzamine</td>
</tr>
<tr>
<td><strong>Sphincteric weakness</strong></td>
<td>Imipramine</td>
</tr>
<tr>
<td></td>
<td>Phenylpropanolamine</td>
</tr>
<tr>
<td></td>
<td>Topical estrogens</td>
</tr>
<tr>
<td></td>
<td>Surgery</td>
</tr>
</tbody>
</table>

Table adapted from Williams and Pannill (1978).
3) Oxybutinin. Oxybutinin has direct antispasmodic activity similar to flavoxate, as well as antimuscarinic and analgesic properties. Its effectiveness in the treatment of detrusor instability is thought to be primarily due to its non-specific musculotropic action, thereby calming detrusor hyperreflexia.

4) Propantheline. Propantheline is a quaternary ammonium compound with potent antimuscarinic activity and ganglionic blocking activity. Thus, propantheline can inhibit excitatory ganglionic transmission as well as the excitatory effect of acetylcholine at the muscarinic receptors of the detrusor and thereby reduces unstable bladder contractions.

5) Bethanechol. Bethanechol is a selective muscarinic receptor agonist, capable of producing bladder contractions in vitro. The effects of bethanechol in vivo enable the bladder to produce a more forceful bladder contraction. It is postulated that an intact reflex pathway is needed for bethanechol to assist in producing a coordinated contraction (Downie, 1981). Bethanechol is therapeutically useful in the treatment of overflow incontinence.

6) Phenoxybenzamine. Phenoxybenzamine is an antagonist at both alpha₁ and alpha₂ adrenoceptors. Antagonism is initially reversible at these sites but later becomes irreversible by covalent bonding to the receptor site. The effects of phenoxybenzamine, therefore, last for days, and dosing intervals can be long. Blockade of alpha adrenoceptors reduces urethral resistance to expulsion, allowing a more
complete detrusor contraction, and thus provides relief from overflow incontinence.

7) Phenylpropanolamine. Phenylpropanolamine is an alpha adrenergic receptor agonist and activates receptors of the urethra to raise the resting urethral pressure, providing greater resistance to loss of urine due to sphincteric weakness.

8) Estrogen. Estrogen therapy is effective treatment for sphincteric weakness which is secondary to atrophic vagina. Estrogen receptors exist in the urethra of women and a deteriorating urethra secondary to atrophic vagina is thought to be the cause of urinary incontinence in these cases.

1.2.2 Research on the aging bladder.

Clinical research. Urinary incontinence affects 10 to 20% of the elderly population but only 2% of the general population. Furthermore, the most common type of incontinence suffered by the elderly is detrusor hyperreflexia, while overflow incontinence and sphincteric insufficiency represent other common forms. Besides architectural or drug-induced causes, geriatric incontinence is often caused by one of the following: CNS defects, alteration of afferent (sensory) pathways, autonomic neuropathy, detrusor or sphincter weakness. With this knowledge of geriatric incontinence, two important questions can be asked:

1) Why is the incidence of incontinence high in the elderly population?
2) Do the causes of geriatric incontinence represent general age-related alterations? That is, are the causes of geriatric incontinence specific for incontinence sufferers, or do these causes represent general declines in bladder function suffered by all elderly which result in incontinence in a small percentage, possibly because of other contributory age-related complications?

To answer these questions, one must examine studies concerned with age-related changes in bladder function in individuals who are not incontinent. Most studies on incontinence include patients suffering from age-related disease states which contribute to incontinence, e.g. bladder diverticula, tumors, prostatic hyperplasia, estrogen deficiency, dementia, etc. Studies on the continent and healthy elderly are valuable examinations of the effect of age alone on bladder function, however, unfortunately few such studies exist and involve small populations of elderly.

Brookehurst and Dillane (1966) performed cystometrographic analyses of 40 non-incontinent elderly women, ages 65 and over, 16 of which had neurological disorders. The cystometrograms of these women were compared to cystometrograms of younger adults. Elderly patients were found to have a decreased bladder capacity, an incomplete voiding contraction, the presence of uninhibited detrusor contractions and a late onset of the desire to micturate. Brookehurst (1979) also reports funneling of the bladder neck as a common condition in the elderly. Thus, the aged bladder, although capable of maintaining continence, was found to be inefficient compared to the younger adult
bladder; the aged bladder does not fill properly, and does not empty properly.

A second and more recent study by Andersen et al. (1978) examined bladder function in 17 healthy elderly males ranging in age from 60 to 75 years (mean age = 66.5 years). No subject had signs of organic neurological disease, determined by an extensive neurological examination, nor a history of incontinence. The most striking observation was the existence of detrusor hyperreflexia in 53% of the patients. Other signs of bladder dysfunction were slow urine streams, frequency, urgency, nocturia and residual urine. Bladder capacity and the onset of the desire to micturate appeared normal for the majority of these patients.

Both Brocklehurst and Dillane (1966) and Andersen et al. (1978) demonstrated detrusor hyperreflexia and residual urine as common findings in aged healthy subjects. Detrusor hyperreflexia is said to be a broad term covering a variety of different problems which have different causes (Malone-Lee, 1982). In fact, the high percentage of detrusor hyperreflexia in incontinent patients with benign prostatic hypertrophy and also with Parkinson's disease has been blamed on obstruction of bladder flow and on supraspinal lesioning of bladder control (Andersen, 1978). In light of the Andersen study, however, detrusor hyperreflexia may be due to age alone, since obstruction and supraspinal lesions were ruled out. Although the questions posed previously cannot be fully answered, the aged bladder does appear to undergo age-related changes that are clearly peculiar to the bladder and not caused by other age-related diseases. Whether detrusor
hyperreflexia and residual urine, when complicated by other contributory age-related diseases, result in geriatric incontinence can only be considered conjectural at the present time.

**Experimental research on the effects of age on the urinary bladder.** To date, only 2 animal studies of the effects of age on the urinary bladder exist. Kolta et al. (1984) examined the effects of age on the response of the whole urinary bladder to autonomic agents using 7, 17 and 29 mo old Fischer 344 (F344) rats (in this laboratory). A significant age-related increase in the responsiveness of the whole bladder to acetylcholine was observed, with a less marked increase in responsiveness to phenylephrine and no observable change in responsiveness to isoproterenol. Age-related changes were observed only in maximum effects of agonists; no change occurred in the ED50 values of any of the agonists. An increase in muscarinic receptor density, measured by \[^3H\]-QNB binding, was found to correlate well with the age-related increase in cholinergic responsiveness. Hayes et al. (1983) also examined the binding of \[^3H\]-QNB to whole bladders of Sprague-Dawley rats, ages 6, 16 and 26 mo. These researchers observed no age-related change in either the density of receptors or the Kd of \[^3H\]-QNB. Hence, there appears to be an age-related change in the responsiveness of the bladder to acetylcholine in F344 rats which may or may not be mediated by a change in the density of muscarinic receptors. Whether these changes can be extrapolated to humans remains to be determined.
CHAPTER II

STATEMENT OF THE PROBLEM

Urinary bladder function is regulated by a complex of nerves, arising from both supraspinal levels and from reflex afferents. These nerves terminate on and regulate the activity of autonomic cell bodies which send motor axons to the smooth musculature of the bladder body and neck. These autonomic nerves, both adrenergic and cholinergic, innervate the bladder and directly influence both filling and expulsion of urine. Control of these two bladder functions is achieved at an early age and is often maintained throughout life. Loss of control, i.e. urinary incontinence, results when proper filling or expulsion is no longer possible because of alterations of the bladder itself, deterioration of the nervous control of the bladder, or other factors which interfere with urine flow (e.g. tumors). Incontinence, thought to be largely the result of neurological deficits, occurs in a large percentage of individuals greater than 65 years of age (10 to 20%) compared to younger humans (2%). Therefore, it is conceivable that changes occur in the nervous control of the bladder with age which predispose the elderly population to urinary incontinence. In fact, studies have shown that the healthy continent elderly have bladder function that is less efficient than bladder function of younger adults. The most striking observation of these studies has been the
high occurrence of unstable detrusor contractions in aged continent bladders.

Experimental research on the aging bladder is lacking. To my knowledge, only one investigation of the effects of age on the bladder in vitro exists (Kolta et al., 1984); this study demonstrated an increase in the responsiveness of the whole bladder of rats to acetylcholine. If these age-effects can be extrapolated to humans, it is conceivable that an age-related increase in the responsiveness of the bladder may underly the unstable detrusor contractions observed in aged human bladders. Of course, this is highly conjectural and demands further experimental and clinical research.

Since urinary bladder function is controlled directly, albeit, in part, by the autonomic nervous system, and since bladder function of the aged is disrupted even in those able to maintain continence, the following hypothesis is put forward: age-related changes in the autonomic control of the urinary bladder may contribute to the age-related disruption of urinary bladder function in the elderly and may ultimately contribute to the development of incontinence.

The goal of this research endeavor has been to investigate the effects of age on the autonomic responsiveness of urinary bladders from aging rats. The findings of Kolta et al. (1984) on the whole bladder are supportive of age-induced alterations in the autonomic control of the bladder. However, filling and expulsion are the result of the coordinated relaxation and contraction of both the bladder body and neck. Therefore, experiments examining the effects of age on the whole bladder fail to answer questions about the two functionally
distinct regions of the bladder. Hence, a major goal of this research is to separately examine the autonomic responsiveness of the two regions of the bladder, determining whether and where age-related changes occur. Emphasis is placed on alpha adrenergic and cholinergic receptor systems to limit the scale of the project.

Specific goals of this research effort are as follows:

1) To identify the effects of age on the functional responsiveness of both the bladder body and neck to the autonomic neurotransmitters, acetylcholine and norepinephrine.

2) To determine what type of cholinergic receptor (nicotinic or muscarinic) and alpha adrenergic receptor (alpha$_1$ or alpha$_2$) mediate contractions in both bladder regions of the F344 rat and to determine which of these receptors is responsible for any age-related alterations in function.

3) To determine whether age-related changes in the responsiveness of a bladder region can be explained by changes in the density or affinity of receptors.
CHAPTER III

METHODS

3.0 Animals

Male albino Fischer 344 (F344) rats of 3 ages were used in all studies. Seven and 16 mo old rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana), and 27 mo old rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Massachusetts). All rats were housed in groups of 4 in stainless steel cages with ground corn cob bedding (Anderson Bed-o-cob) and were provided free access to Purina rat chow (No. 5012) and tap water. The animal facility was maintained at 21°C and on a 12 hour light-dark cycle (on-off at 0600 and 1800).

The mean age of death for the male F344 rat is 27.5 months (Sass et al. 1975). Therefore, it is not surprising that the group of 27 mo old rats suffer from a number of age-related problems. For example, these rats are typically more susceptible to death due to respiratory disease and are often plagued with tumors. In these studies, the aged rat was examined for any abnormality that could possibly affect urinary bladder function, e.g. a large tumor which could obstruct urinary flow or hypoxia due to severe pneumonia. Animals with severe pneumonia or with large tumors anywhere in the abdominal region were not used.
3.1 Isolated Bladder Preparations

3.1.1 Preparation of bladder regions.

Bladder tissues were isolated from rats of the 3 age groups by the method of Hukovic et al. (1965). Rats were killed by a sharp blow on the head and bled. The lower abdomen was opened with a rostrocaudal incision extending from the sternum to the penis. The bladder was located, and the connective tissue surrounding the bladder was dissected away. The prostate gland, connective tissue and fat surrounding the base of the bladder were also carefully removed. A thread was attached by passing a needle through the rostral tip of the bladder muscle; the bladder body (detrusor muscle) was then dissected with a lateral cut at precisely the level of the ureters. A second thread was attached to the lower end of the detrusor, and the tissue was mounted in a 10 ml jacketed organ bath. Another thread was attached to the most proximal part of the bladder remaining in the animal. The bladder neck muscles (deep trigone, superficial trigone and proximal urethra) were excised together with a second cut made at the level of the pelvis, just distal to the external urethral sphincter. The overlying skeletal muscle comprising the external urethral sphincter and fatty tissue were removed with great care as not to cut the underlying smooth muscle tissue. The remaining bladder neck smooth muscle was cut spirally and suspended in an organ bath after attaching a thread to the distal end of the neck region. Resting tension for the bladder body and the bladder neck was 1.5 g and 0.3 g, respectively. Both tissues were bathed in a physiological salt solution (PSS) of the following composition (mM): NaCl, 120.7; KCl, 5.9; CaCl$_2$$ \cdot 2$H$_2$O, 1.25;
MgCl$_2$·6H$_2$O, 1.2; NaHCO$_3$, 15.5; NaH$_2$PO$_4$·H$_2$O, 1.2; and glucose 11.5; dissolved in double distilled demineralized water. The tissues were maintained at 37° C in PSS aerated with 95% O$_2$ and 5% CO$_2$. Tissues were permitted to equilibrate for 45 minutes prior to the addition of drugs and were washed with PSS at 20 minute intervals throughout each experiment. Muscle contractions were recorded isotonically on a physiograph recorder (Narco-Biosystems, DMP-4B) using a lever system with isotonic myograph detector heads and isotonic myograph transducers (Narco-Biosystems, MK II, Ser. 495 and Ser. 1287).

3.1.2 Experimental procedure.

Concentration-response data. One bladder body and bladder neck from each of the 3 age groups were tested in parallel for their responses to identical drugs. The tissues were exposed to a single submaximal concentration (30 μM) of either a cholinergic or an alpha adrenergic receptor agonist. This initial submaximal contraction stabilized the tissue and allowed for more consistent results in the subsequent concentration-response (CR) determinations obtained. Following several washes after this drug addition, CR data were obtained by cumulative addition of drugs, unless otherwise indicated. Successive additions of drug to the baths were performed at intervals that allowed the tissue to contract maximally at a given drug concentration. This interval varied somewhat from one agonist to another, but was generally between 0.5 to 3 minutes. When possible, two successive CR curves to a given agonist (separated by a suitable washout period) were averaged. No single tissue was subjected to more than one agonist (except when indicated) so that the length of time the tissue was
used was minimized, avoiding tissue fatigue. CR data for phenylephrine and clonidine were obtained on the same tissue and were the only exception to "one tissue--one agonist"; the phenylephrine CR was obtained first. Responses to acetylcholine were carried out in tissues continuously incubated with the acetylcholinesterase inhibitor, physostigmine (30 μM). Responses to phenylephrine and clonidine were obtained by preincubating the tissues with propranolol (0.1 μM) for 5 to 10 minutes. The concentration of propranolol used to obtain norepinephrine CR data varied as indicated in RESULTS. Propranolol served to eliminate any beta adrenergic agonist activity of these alpha agonists.

After agonist-induced responses were obtained, the tissues were washed repeatedly and allowed to relax 30 to 60 minutes, until restoration of pre-drug baseline tissue lengths. Each tissue was then subjected to 100 mM KCl, then 300 mM KCl, separated by suitable washout periods. (In previous experiments investigating a wide range of KCl concentrations, it was demonstrated that bladder tissues from rats of the 3 age groups always contracted the greatest in the presence of 100 mM and/or 300 mM KCl.) The maximum KCl contraction was then computed as the larger of the 2 contractions induced by KCl, in mm tissue movement. Individual tissue responses to agonists were standardized by expressing responses as a percentage of the maximum contraction (100%) induced by KCl. As an alternate method of standardization, tissue responses were normalized to the weight of the tissue using the following equation:

\[
\text{response} = \frac{\text{mm of tissue movement}}{\text{wet weight tissue (g)}}
\]
Occasionally, only the mean weight of a group of tissues used to investigate an agonist was known. In these cases, response was computed as:

\[
\text{mm/g tissue} = \frac{\text{mm of tissue movement}}{\text{mean wet weight tissue (g)}}
\]

Uptake experiment. This experiment was designed to examine whether neuronal and non-neuronal uptake processes affect norepinephrine CR data in the bladder tissues and to determine the effect of aging on these uptake systems. Cocaine HCl (100 \( \mu \)M) and hydrocortisone sodium phosphate (30 \( \mu \)M) were used to block uptake\(_1\) (neuronal uptake) and uptake\(_2\) (non-neuronal uptake), respectively. Preliminary studies demonstrated that hydrocortisone had no effect the CR curves of norepinephrine in either bladder region but was added to the baths in these experiments to be certain that the uptake\(_1\) process was blocked. Propranolol (100 \( \mu \)M, bladder neck; 300 \( \mu \)M, bladder body) was used to antagonize the action of norepinephrine at beta adrenoceptors. Tissues were subjected to 2 cumulative norepinephrine additions, the first in the presence of propranolol alone, and the second in the presence of propranolol, cocaine and hydrocortisone. Finally, maximum contractions of the tissues to KCl were determined in the absence of any drug.

Determination of \( pA_2 \) values. Determination of \( pA_2 \) values of prazosin and yohimbine were performed as described by Arunlakshana and Schild (1959). Bladder tissues were isolated and mounted as described above and continuously bathed in PSS containing cocaine (10 \( \mu \)M) and propranolol (1 \( \mu \)M). Each bladder region of each age group was
subjected to 5 cumulative concentration responses to phenylephrine, the first in the absence of an alpha adrenoceptor antagonist, and the next 4 in the presence of cumulatively increasing concentrations of either prazosin, an alpha₁ selective antagonist, or yohimbine, an alpha₂ selective antagonist. No maximum contraction to KCl was obtained in these experiments; each agonist CR was computed as a percentage of the maximum contraction elicited by the agonist when in the presence of the particular concentration of antagonist.

3.1.3 Data analysis.

Concentration-response data. Pen movement recorded by the physiograph was measured using a Complot Series 7000 Digitizer (Houston Instruments) and converted to mm of tissue movement using the S.A.S. program, COMPLOT (APPENDIX A). Data obtained from COMPLOT was in the form necessary for input into the ITON1, ITON2 and KITSTAT programs. (APPENDIX B, D and E, respectively). The CR data (in mm tissue movement) computed by COMPLOT were fit to a hyperbolic logistic function (Parker and Waud, 1971) by a method of non-linear least squares regression in ITON1. Hence, ITON1 fits concentration-response data to the following hyperbolic equation:

\[
\text{Effect} = \frac{E_{\text{max}} \times (\text{drug})^P}{(\text{drug})^P + K^P}
\]

where \(E_{\text{max}}\) is the maximum contraction induced by a drug, \(K\) is the concentration of agonist necessary to produce a response that is 50% of the \(E_{\text{max}}\), and the exponent \(p\) describes the shape of the CR curve. \(E_{\text{max}}\) and \(K\) values were adjusted through the iterative procedure until a satisfactory fit of the data was found. ITON1 also plots effect
versus concentration for the original data and for the predicted values computed by fitting the data to the hyperbolic logistic function. Thus, the closeness of fit of the derived values to the actual data could be visualized (APPENDIX C). The Emax values were then expressed as either percentages of the maximum contraction to KCl or as mm tissue movement/g wet weight tissue. ITON2 was then used to compute effects as a percentage of the maximum KCl-induced contraction for that tissue and to compute concentrations necessary to produce 10, 20, 30, ...100% effects as ED10, ED20, ED30, ...ED100, respectively, by logarithmic interpolation. ED50 values for each concentration-response curve were computed by replacing the maximum KCl-induced contraction by the Emax computed by ITON1 and then subjecting the data to ITON2. (This could also be accomplished in ITON1 by adding a few lines to the program for an output of K values). Thus, ED50 is defined here as the concentration of drug necessary to produce a response that is 50% of the maximum drug-induced response for that tissue. Means and standard errors of the means of data expressed as percentages of the maximum KCl-induced contraction were computed using KITSTAT.

\[ pA_2 \text{ computations.} \] Values for the \( pA_2 \) of prazosin and yohimbine were calculated according to Arunlakshana and Schild (1959) according to the following equations:

\[ \frac{A'}{A} = 1 + \frac{B}{K_B} \quad (2) \]

and

\[ pA_2 = -\log K_B \quad (3) \]

where \( \frac{A'}{A} \) is the dose-ratio (DR) for the effect in the presence of
the antagonist, B (A'), and in the absence of the antagonist (A). $K_B$ is the dissociation constant of the antagonist. Rearrangement of equation (3) gives:

$$\log (DR - 1) = \log B - \log K_B$$  \hspace{1cm} (4)

A plot of log (DR-1) vs. log B produces a straight line with the slope of unity (for a single site) and an abscissa intercept equal to the $pA_2$.

3.2 Muscarinic Receptor Binding Using $^3$H-QNB

3.2.1 Tissue preparation.

Binding assays were performed as described by Yamamura and Snyder (1974) with some modification. Initial experiments were performed on bladder tissues that were used in tissue bath experiments examining the functional response of the cholinergic receptor system. These tissues had been frozen immediately following removal from the baths over one year prior to the binding experiments. Using these tissues, binding was erratic and impossible to interpret. Subsequently, fresh frozen bladder tissues, frozen for not more than 3 months and not previously used in any experiments, were used to examine the effects of age on $[^3H]$-quinuclidinylbenzilate (QNB) binding. These tissues were isolated as described for tissue bath experiments and binding to bladder regions of each age group was run in parallel on each day of experimentation. After thawing, tissues were blotted dry for weighing, then placed immediately in ice cold Na$^+$-phosphate buffer (50 mM, pH 7.4 at 25°C) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). Tissues were then dissected free of any fat or connective
tissue, finely minced with a pair of scissors and placed into ice cold homogenizing tubes. Two detrusors were pooled (total wet weight of 100 to 200 mg), then homogenized in 15 ml of Na\(^+\)-phosphate/PMSF buffer with a Brinkman Polytron (setting 10; 2-15 sec bursts separated by 5 min in ice) and by 15 seconds with a hand homogenizer. The homogenate was then diluted further to 32 ml. Three bladder necks were pooled (total wet weight of 60 to 100 mg), homogenized and diluted to a volume of 28 ml. This procedure was followed for tissues of each age group, and allowed the determination of mg tissue, mg protein, and fraction of bladder region per incubation vial. Binding reaction mixtures contained 1 ml of the homogenate, \(^3\)H-QNB (30.2 Ci/mmol) and, when determining non-specific binding, atropine sulfate (1 \(\mu\)M), in a total volume of 1.5 ml Na\(^+\)-phosphate/PMSF buffer. The reaction was initiated by the addition of the homogenate and the mixture was incubated for 60 min at 25\(^\circ\) C in a shaking incubator. Binding was terminated by suction filtration on glass fiber filter papers (Whatman GF/B, 2.4 cm previously soaked in 1% polyethyleneimine for 60 min). Filters were washed with 15 ml ice cold Na\(^+\)-phosphate buffer and radioactivity was counted in a Beckman LS-6800 at a counting efficiency of 30 to 35%. Non-specific binding was calculated as the difference between binding in the absence and presence of atropine. Saturation curves for \(^3\)H-QNB binding to its receptor were generated by incubating tissues with increasing concentrations of \(^3\)H-QNB (0.03 to 1.55 nM). Saturation data were analyzed by linear regression of bound/free vs. bound (Scatchard, 1949) using the S.A.S. program, SCATCHARD (APPENDIX F).
3.2.2 Protein assay.

Tissue homogenates were not entirely soluble in the standard Lowry assay solutions. Hence, tissue homogenates were boiled in an equal volume of 1 N NaOH for 20 minutes in a boiling water bath to dissolve protein. Even after this vigorous method, small amounts of undissolved tissue were noted in the bottom of bladder homogenate tubes. Even so, 50 to 100 µl of these NaOH treated homogenates were assayed for protein by the method of Lowry et al. (1951) using an identically treated bovine serum albumin standard.

3.3 Alpha Adrenoceptor Binding

3.3.1 \[^{125}\text{I}]\text{[1-2-}[\text{beta-(4-hydroxyphenyl)}-\text{ethyl-aminomethyl}\text{-tetralone (HEAT) binding.}]

Demonstration of specific alpha adrenoceptor binding in the urinary bladder tissues is made difficult because of the low density of receptors and the lack of a ligand with both a high specific activity and a low amount of non-specific binding. Initial experiments involved in examining alpha receptor binding were attempted utilizing the selective alpha\textsubscript{1} antagonist, \[^{125}\text{I}]\text{-HEAT (2200 Ci/mmol). Several experiments were performed to optimize conditions for \[^{125}\text{I}]\text{-HEAT binding to bladder tissues. Three bladder bodies were isolated, minced with scissors, and homogenized in 15 ml buffer with a Brinkman Polytron (setting 10; 2-15 sec bursts separated by 5 min in ice) and by 15 seconds with a hand homogenizer. The buffer used was 50 mM Tris-HCl, 154 mM NaCl, 1.0 mM ascorbic acid, supplemented with 100 µM PMSF (pH = 7.4 at 37° C.). The homogenate was then passed through 2 layers of gauze, followed by centrifugation of the filtrate at}
39,000 X g for 20 minutes. The supernatant was discarded, 25 ml of fresh buffer was added to resuspend pellet, and the homogenate was centrifuged again. After discarding the supernatant, the pellet was resuspended in 8 ml of buffer. This method of preparation was necessary to decrease the non-specific binding of $^{125}$I-HEAT to filters. The binding reaction mixture contained 200 μl of homogenate, ligand and 10 μM phentolamine to define non-specific binding (total volume = 300 μl). Under these conditions, in some cases specific $^{125}$I-HEAT binding could be demonstrated; however, binding was erratic and was dependent on either ligand or tissue concentration. Hence, attempts at saturation analysis of $^{125}$I-HEAT to bladder tissues failed. It was found that $^{125}$I-HEAT bound to reaction cups, pipet tips and even the metal manifold at concentrations of $^{125}$I-HEAT necessary for saturation of receptor binding (1-5 nM). Binding of $^{125}$I-HEAT to glass and plastic has been previously described (Glossman et al., 1981). Also binding of $^{125}$I-HEAT to sites only displaceable by cold HEAT, and not displaceable by prazosin and phentolamine has been described (Adams and Jarrott, 1984), thus increasing the total amount of non-specific binding of this ligand. Given this and the problems faced in the above experiments, I concluded that $^{125}$I-HEAT is not a suitable ligand for alpha receptor binding experiments in urinary bladder tissues.

3.3.2 $^{3}$H dihydroergocryptine (DHE) binding.

Measurement of alpha adrenoceptor densities was also attempted using the ligand, $^{3}$H DHE, for which binding to rabbit urinary bladder tissues has been previously described (Larsson, et al., 1981).
My attempt to demonstrate specific binding of $^3$H-DHE to the bladder body failed for one or more of the following possible reasons. (1) The specific activity of the $^3$H-DHE (20 Ci/mmol) used in the present study was probably too low to detect the alpha adrenoceptors, which are probably sparsely located. (2) The density of alpha adrenoceptors may be greater in bladder of the rabbit (used by Larsson, 1983) than in the F344 rat. (3) Of course, it is also possible that conditions were not appropriately optimized for $^3$H-DHE binding to rat urinary bladder tissues. Nevertheless, it was concluded that $^3$H-DHE (20 Ci/mmol) was not a suitable ligand for alpha receptor binding to F344 rat bladder tissues.

3.4 Statistical Analysis

$E_{max}$ (maximum drug-induced contraction computed by ITON1) and $B_{max}$ (total number of receptors computed by Scatchard analysis of saturation binding) values of the three age groups were statistically analyzed using the Duncan's multiple comparisons test (Sokal and Rohlf, 1969) computed with the aid of the ANOVAR program (Appendix G). Differences between mean ED50 values and mean Kd (dissociation constant calculated by SCATCHARD) values were considered significant when their 95% confidence intervals (C.I.) did not overlap. The Student's t test was used (Sokal and Rohlf, 1969) when comparisons were made between only 2 groups of data.

3.5 Drugs

All drugs used in the tissue bath experiments were dissolved in
PSS on a daily basis. Drugs, drug sources and solvents used to dissolve the drugs are listed in Table 4. All radionuclides used, their sources and the specific activity of each are also listed in Table 4.
## TABLE 4

Drugs, Chemicals, Radionuclides, and Solvents Used

<table>
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<tr>
<th>Drugs and Chemicals</th>
<th>Source</th>
<th>Solvent</th>
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</thead>
<tbody>
<tr>
<td>Acetylcholine HCl</td>
<td>Sigma</td>
<td>PSS(^a)</td>
</tr>
<tr>
<td>Atropine sulfate</td>
<td>Sigma</td>
<td>PSS</td>
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<tr>
<td>Bethanechol HCl</td>
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<td>Clonidine HCl</td>
<td>Boehringer Ingelheim</td>
<td>PSS</td>
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<tr>
<td>Cocaine HCl</td>
<td>Merck, Sharp &amp; Dome</td>
<td>PSS</td>
</tr>
<tr>
<td>Hydrocortisone sodium</td>
<td>Merck, Sharp &amp; Dome</td>
<td>PSS</td>
</tr>
<tr>
<td>l-Norepinephrine HCl</td>
<td>Sigma</td>
<td>PSS + AA(^b)</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>Sigma</td>
<td>PSS</td>
</tr>
<tr>
<td>Pilocarpine HCl</td>
<td>Sigma</td>
<td>PSS</td>
</tr>
<tr>
<td>l-Phenylephrine HCl</td>
<td>Sigma</td>
<td>PSS + AA</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>Boehringer Mannheim</td>
<td>0.5 ml ethanol</td>
</tr>
<tr>
<td>Physostigmine HCl</td>
<td>Sigma</td>
<td>PSS</td>
</tr>
<tr>
<td>Prazosin HCl</td>
<td>Ayerst</td>
<td>dist. H(_2)O</td>
</tr>
<tr>
<td>d,1-Propranolol HCl</td>
<td>Ayerst</td>
<td>dist. H(_2)O</td>
</tr>
<tr>
<td>Tris base</td>
<td>Sigma</td>
<td>dist. H(_2)O</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Sigma</td>
<td>dist. H(_2)O</td>
</tr>
<tr>
<td>Yohimbine HCl</td>
<td>Sigma</td>
<td>dist. H(_2)O</td>
</tr>
<tr>
<td>(^{3})H]-QNB (30.2 Ci/mmol)</td>
<td>New England Nuclear</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>(^{3})H]-DHE (20.0 Ci/mmol)</td>
<td>New England Nuclear</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>(^{125})I]-HEAT (2200 Ci/mmol)</td>
<td>New England Nuclear</td>
<td>Tris buffer</td>
</tr>
</tbody>
</table>

\(^a\) Physiological salt solution (see Appendix 1).

\(^b\) Physiological salt solution containing 30 mM ascorbic acid.
CHAPTER IV

RESULTS

4.0 Overview

This chapter is divided into three general sections: 1) general observations on the aging bladder, 2) effects of age on the cholinergic receptor system, and 3) effects of age on the alpha adrenergic receptor system. Within each section are subsections of observations on the bladder body and the bladder neck.

4.1 General Observations on the Aging Bladder

Although it was difficult to visually distinguish 7 mo old F344 rats from 16 mo old rats, the 27 mo old rats had several distinctive characteristics: whitish eyes (presumably from cataracts), yellowed hair, long toenails, usually an enlarged spleen, and always grossly necrotic testes. The general appearance of the bladder neck in rats of all three ages did not differ. The bladder body muscle grossly appeared to be thicker and more vascularized in the 27 mo old rat compared to the 7 mo old rat. Several general observations on bladder tissues from rats of the 3 age groups were quantitated and are summarized in Table 5. Changes in 16 and 27 mo tissues with respect to the 7 mo tissues in terms of whole body weight, tissue weight, percent
### TABLE 5

**Effects of Age on the Weight of the F344 Rat and on Various Parameters of the Urinary Bladder Regions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body weight (g)</td>
<td>32</td>
<td>364±5</td>
<td>422±5*</td>
<td>409±5*</td>
</tr>
<tr>
<td>Bladder body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet weight (mg)</td>
<td>30</td>
<td>61.8±1.7</td>
<td>73.0±2.5</td>
<td>94.5±2.7</td>
</tr>
<tr>
<td>dry weight (mg)</td>
<td>10</td>
<td>11.1±0.4</td>
<td>13.8±0.5</td>
<td>16.2±0.6</td>
</tr>
<tr>
<td>% water</td>
<td>10</td>
<td>73.1±0.3</td>
<td>72.5±1.3</td>
<td>73.6±0.8</td>
</tr>
<tr>
<td>% protein</td>
<td>4</td>
<td>34.8±1.1</td>
<td>27.3±5.6</td>
<td>28.5±3.2</td>
</tr>
<tr>
<td>KCLmax (mm)</td>
<td>32</td>
<td>13.9±0.5</td>
<td>13.2±0.5</td>
<td>14.5±0.6</td>
</tr>
<tr>
<td>KCLmax (mm/g)</td>
<td>4</td>
<td>190±18</td>
<td>171±8</td>
<td>152±5</td>
</tr>
<tr>
<td>Bladder neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet weight (mg)</td>
<td>32</td>
<td>29.9±1.1</td>
<td>34.1±1.5</td>
<td>34.0±1.7</td>
</tr>
<tr>
<td>dry weight (mg)</td>
<td>10</td>
<td>6.50±0.47</td>
<td>8.06±0.63</td>
<td>6.97±0.63</td>
</tr>
<tr>
<td>% water</td>
<td>10</td>
<td>68.7±0.7</td>
<td>68.7±0.5</td>
<td>68.8±0.5</td>
</tr>
<tr>
<td>% protein</td>
<td>4</td>
<td>13.0±1.9</td>
<td>9.82±0.9</td>
<td>9.78±1.3</td>
</tr>
<tr>
<td>KCLmax (mm)</td>
<td>36</td>
<td>3.66±0.16</td>
<td>4.59±0.23</td>
<td>3.90±0.22</td>
</tr>
<tr>
<td>KCLmax (mm/g)</td>
<td>10</td>
<td>222±15</td>
<td>191±21</td>
<td>177±15</td>
</tr>
</tbody>
</table>

Table 5 continued on page 62.
Table 5. (continued)

Data shown are means ± S.E.M. and were collected from tissues used in both tissue bath and binding experiments.

a Dry weight and percent water was obtained by determining wet weight of the tissue, drying the tissue for 7 days at 60°C and then re-weighing.

b \[% \text{ water} = \frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100\%\].

c Values for % protein were calculated as: mg protein/mg tissue x 100%. These values are means ± S.E.M. for 4 separate determinations made on tissue homogenates used in binding experiments.

d KClmax in mm is the mean of the KClmax values of pooled data from all tissue bath experiments, while KClmax in mm/g is the mean of only 4 to 8 determinations.

* Significantly greater than 7 mo group (p > 0.05).

** Significantly greater than 7 mo group (p > 0.01).
protein, and percent water are depicted in Figures 5 and 6. A significant increase in the weight of the rat occurred in 16 and 27 mo rats compared to the 7 mo rat. Also significant changes in the wet and dry weights occurred in the bladder body from 7 to 16 mo and from 16 to 27 mo (p < 0.01, Duncan's). No other significant age-related changes were observed in weight, percent water or percent protein. While the change in the weight of the bladder neck follows closely the change in the weight of the whole animal, the weight change in the bladder body exceeds that of the whole animal. Furthermore, Figures 5 and 6 show that in both bladder regions, an increase in weight occurs with a decrease in the percent protein which cannot be explained by a change in the percent water. Therefore, the increase in weight must partly be a result of an increase in non-protein material or an increase in protein that is no longer detectable by the Lowry protein assay (see DISCUSSION). An attempt was made to determine any change in muscle quantity; however, biochemical quantitation of smooth muscle by examining myosin ATP-ase activity was unsuccessful.

Values for the maximum contractions induced by the non-specific contractile agent KCl (KClmax) appear in Table 5. No significant age-related change in the KClmax expressed in mm was observed in the bladder body (Figure 7). In the bladder neck, the KClmax of the 16 mo group was 25 and 18% greater (p < 0.05, Duncan's) than the 7 and 27 mo groups, respectively. While no significant changes occur in either bladder region when the KClmax values are expressed as mm/g tissue, gradual declines in the responsiveness of both regions are evident. Since KClmax, expressed as mm tissue movement, showed no consistent
Figure 5. The percentage change in rat weight and in various parameters of the bladder body with age.

Percentage changes in the parameters are calculated as percentage changes from the values of the 7 mo group and are depicted as rat weight, — — —; wet weight of bladder body, + — —; dry weight of bladder body, — — —; percent water, O — —; percent protein, X — —. The greatest change with age occurred in the weight of the bladder body, which exceeded the increase in the weight of the whole animal.
Figure 5

The graph shows the percent change over age (in months) from 27 to 7 months. The x-axis represents age in months, and the y-axis represents the percent change. There are multiple lines with different symbols, indicating different groups or conditions, which are not labeled in the image.
Figure 6. The percentage change in the rat weight and in various parameters of the bladder neck with age.

Percentage changes in the parameters are calculated as percentage changes from the values of the 7 mo group and are depicted as rat weight, •—•; wet weight of bladder neck, +——+; dry weight of bladder neck, *—*; percent water, ○——○; percent protein, Y—Y. A change in the weight of the bladder neck with age was paralleled by a similar change in the weight of the whole animal. The most marked age-related change occurred in the percent protein, which underwent nearly a 30% decrease.
Figure 6
Figure 7. Maximum contractions induced by KCl (KClmax) in the bladder body and neck from rats of three age groups.

Bladder tissues were isolated from rats of ages 7 mo (open bars), 16 mo (diagonal bars) and 27 mo (hatched bars). Maximum contractions occurred at bath concentrations of 0.1 to 0.3 mM KCl. Data are means ± S.E.M. for 32 to 36 tissues per age group. No consistent age-dependent changes in the KClmax were observed. ** indicates significantly greater than both 7 and 27 mo groups (p < 0.05).
Figure 7
age-dependent change in either bladder region, it provided a standard by which age-induced changes in the autonomic responsiveness could be examined.

4.2 The Effects of Age on the Cholinergic Receptor System

4.2.1 Functional studies.

The purpose of these studies was to verify and extend the findings of Kolta et al. (1984) and to determine whether age affected the cholinergic responsiveness of both the bladder body and neck. CR curves were obtained for the neurotransmitter acetylcholine, the selective muscarinic receptor agonists bethanechol and oxotremorine, and the cholinergic partial agonist pilocarpine in the two bladder regions from 7, 16 and 27 mo old rats (Figures 8-11). These curves were analyzed as described in METHODS and compared in terms of the Emax and ED50. In the bladder neck, 31%, 56%, 60% and 123% increases in Emax values (7 vs. 27 mo of age) were observed with age for pilocarpine, acetylcholine, bethanechol and oxotremorine, respectively. The greatest increase in responsiveness of the neck occurred between the ages of 16 and 27 mo (with the exception of acetylcholine). In contrast to the age-related changes in the bladder neck (Figure 13, Table 6), no changes in Emax were observed in the bladder body (Figure 12, Table 6). Although age comparisons of response are made at the Emax, age-related increases in responsiveness are obvious at concentrations of agonist much lower than that needed to induce a maximum response (Figures 8-11). The pD₂ values (pD₂ = - log molar ED50) for all drugs acting on both bladder regions obtained from the three age
Figure 8. Cumulative concentration-response curves for acetylcholine-induced contractions of bladder body and neck tissues from 3 age groups.

Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 to 5 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 8
Figure 9. Cumulative concentration-response curves for bethanechol-induced contractions of bladder body and neck tissues from 3 age groups.

Age groups are designated as 7 mo (▲); 16 mo ( ■ ); and 27 mo ( ● ). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 10. Cumulative concentration-response curves for oxotremorine-induced contractions of bladder body and neck tissues from 3 age groups.

Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 10

PERCENT OF KCl CONTRACTION

BODY

NECK

LOG MOLAR [OXOTREMORINE]
Figure 11. Cumulative concentration-response curves for pilocarpine-induced contractions of bladder body and neck tissues from 3 age groups.

Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 11
Figure 12. Maximum contractions (Emax) of bladder body tissues of 3 age groups to acetylcholine (ACH), bethanechol (BETH), oxotremorine (OXOT) and pilocarpine (PILO).

Age groups are designated as: 7 mo (open) 16 mo (diagonal) and 27 mo (hatched). Each bar represents the mean ± S.E.M. of 4 to 5 tissues of each age group. Maximum contractions to muscarinic agonists are expressed as a percentages of the maximum KCl-induced contraction of each tissue. No significant differences between age groups were observed.
Figure 12
Figure 13. Maximum contractions (Emax) of bladder neck tissues of 3 age groups to acetylcholine (ACH), bethanechol (BETH), oxotremorine (OXOT) and pilocarpine (PILO).

Age groups are designated as: 7 mo (open) 16 mo (diagonal) and 27 mo (hatched). Each bar represents the mean ± S.E.M. of 4 to 5 tissues of each age group. Maximum contractions to muscarinic agonists are expressed as percentages of the maximum KCl-induced contraction of each tissue. * indicates a significant difference from 7 mo group (p<0.05). ** indicate significant differences from 7 and 16 mo groups (p<0.05). A consistent age-related increase in the Emax of all muscarinic agonists was observed.
Figure 13

PERCENT OF KCL CONTRACTION

ACH

BETH

OXOT

PILO

*
## TABLE 6

Maximum Contractions (Emax) Induced by Muscarinic Agonists in the Bladder Body and Neck Regions from Rats of 3 Age Groups

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Tissue</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>body</td>
<td>89.2±4.4</td>
<td>91.5±3.0</td>
<td>98.5±3.4*</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>47.5±8.0</td>
<td>62.2±4.3</td>
<td>74.2±5.8</td>
</tr>
<tr>
<td>Bethanechol</td>
<td>body</td>
<td>96.4±5.5</td>
<td>105.5±1.9</td>
<td>106.5±16.6**</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>52.4±2.9</td>
<td>61.6±4.6</td>
<td>83.6±6.0</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>body</td>
<td>87.9±4.8</td>
<td>86.3±2.9</td>
<td>84.0±7.0*</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>21.9±3.6</td>
<td>25.3±5.1</td>
<td>49.0±3.1</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>body</td>
<td>63.6±3.8</td>
<td>73.8±3.3</td>
<td>74.3±4.4</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>24.8±4.6</td>
<td>21.4±2.4</td>
<td>32.6±4.8</td>
</tr>
</tbody>
</table>

Emax values are expressed as percentages of the maximum KCl-induced contraction. Each value represents the mean ± S.E.M. for 4 to 5 bladder tissues per age group.

* Significantly greater than 7 mo group (p < 0.05).

** Significantly greater than both 7 and 16 mo groups (p < 0.05).
groups are shown in Table 7; no significant age-related changes were observed although higher affinities of the agonists for the bladder body muscarinic receptors is evident for all agonists in all age groups.

The possibility that an increase in the contraction may simply result from a larger tissue was examined. Normalization of bladder neck contractions, so as to account for the weight of the tissue, i.e. expressing contractions as mm tissue movement per g wet weight of tissue, gave nearly identical results as normalization of the contractions to the maximum KCl-induced contraction (Figure 14). This is not surprising since the weight of this tissue does not undergo an age-dependent change. The bladder body weight increases significantly with age, but expression of oxotremorine-induced contractions as mm/g wet weight for the bladder body did not result in the appearance of any significant age-related change (Figure 15), although a moderate 25% (7 mo vs. 27 mo) decline in responsiveness appears. An age-related 12% (7 mo vs. 27 mo) decline in Emax values for bethanechol was also observed, but was not statistically significant (Table 8).

Oxotremorine produced maximum contractions 1/3 to 1/2 the magnitude of those of acetylcholine and bethanechol in the bladder neck, whereas oxotremorine appeared to be a full agonist in the bladder body (Table 6). Since the rate of contraction induced by oxotremorine was observed to be slower than the other agonist-induced contractions, the possibility that the bladder neck became desensitized during its incubation with oxotremorine was examined. Maximum contractions of bladder necks from 7, 16 and 27 mo old rats were induced by a single high
Figure 14. Cumulative concentration-response curves for oxotremorine-stimulated contractions of bladder necks from 3 age groups.

Contractions are expressed as percents of maximum KCl-induced contractions for each tissue (A) and as mm tissue movement per g wet weight tissue (B). Age groups are designated as 7 mo (∆); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each age group. Normalization of contractions to the weight of the tissue do not effect the magnitude of age-dependent changes.
Figure 14
Figure 15. Cumulative concentration-response curves for oxotremorine-stimulated contractions of bladder body tissues from 3 age groups.

Contractions are expressed as percents of maximum KCl-induced contractions for each tissue (A) and as mm tissue movement per g wet weight tissue (B). Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each age group. A moderate decrease in muscarinic responsiveness appears when the data are normalized to the weight of the bladder body.
TABLE 7

*pD*₂ Values of Muscarinic Agonists in Bladder Tissues from Rats of 3 Age Groups

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Tissue</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>body</td>
<td>6.35±0.09</td>
<td>6.26±0.21</td>
<td>6.36±0.05</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>5.33±0.14</td>
<td>5.71±0.18</td>
<td>5.47±0.25</td>
</tr>
<tr>
<td>Bethanechol</td>
<td>body</td>
<td>5.14±0.10</td>
<td>5.26±0.09</td>
<td>5.03±0.08</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>4.70±0.07</td>
<td>4.83±0.07</td>
<td>4.78±0.07</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>body</td>
<td>6.82±0.13</td>
<td>6.88±0.10</td>
<td>6.69±0.18</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>6.59±0.20</td>
<td>6.70±0.07</td>
<td>6.59±0.08</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>body</td>
<td>5.59±0.06</td>
<td>5.75±0.03</td>
<td>5.66±0.04</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>5.29±0.06</td>
<td>5.36±0.08</td>
<td>5.37±0.09</td>
</tr>
</tbody>
</table>

Each *pD*₂ value (*pD*₂ = -log ED50) represents the mean ± S.E.M. for 4 experiments. ED50 values were determined by calculating the concentration of agonist necessary to elicit a contraction 1/2 the magnitude of the maximum obtainable contraction elicited by that agonist in the tissue. No significant differences were observed, judged by the overlap of the 95% C.I. of the means.
TABLE 8

Maximum Contractions (Emax) Induced by Muscarinic Agonists in the Bladder Body of 7, 16 and 27 mo Rats Normalized to Weight

<table>
<thead>
<tr>
<th>Agonist</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>230±30</td>
<td>194±28</td>
<td>224±14</td>
</tr>
<tr>
<td>Bethanechol</td>
<td>276±50</td>
<td>278±19</td>
<td>243±56</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>192±31</td>
<td>208±12</td>
<td>144±28</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. for 4 to 5 tissues per age group. Individual Emax values were calculated by dividing the maximum agonist-induced contraction in mm by the mean weight of the 4 to 5 tissues of that age group. No significant differences were observed.
concentration of oxotremorine (10 µM), followed by a cumulative CR and finally another single-concentration maximum response with repeated washings between single and cumulative additions (Table 9). Maximum contractions induced by single high concentrations of oxotremorine were comparable to those induced by the cumulative addition of the full agonists, acetylcholine andbethanechol. These results confirmed that the continued presence of oxotremorine during a cumulative drug addition induced desensitization of the bladder neck smooth muscle. Furthermore, the single concentration method of Emax determination demonstrated age-related changes similar to those observed by the cumulative addition of acetylcholine andbethanechol (Tables 6 and 9).

The effect that desensitization can have on CR data is depicted in Figure 16. The time to reach a full contraction at each successive concentration is much longer for oxotremorine than for the other muscarinic agonists (5-10 min for oxotremorine vs. 0.5 to 2 min for other agonists tested). Hence, in the initial experiments designed to investigate muscarinic responsiveness of the bladder neck to oxotremorine, the time between successive concentrations of oxotremorine was not sufficient for full contractions to occur at each concentration (1-3 minutes). CR curves for oxotremorine that appear in Figure 10 were the result of 5-10 minute periods between successive oxotremorine concentrations, allowing a full contractions at each concentration. The results of the experiment with only 1-3 min intervals between successive concentrations were misleading in that no age-related increase in muscarinic responsiveness was apparent (Table 9, see cumulative--uncontrolled). These results indicate that desensitization must occur
TABLE 9

Effects of Age on Maximum Contractions Elicited by Acetylcholine and Oxotremorine in the Bladder Neck

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Maximum Contraction (% KCl_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 mo</td>
</tr>
<tr>
<td>Oxotremorine (1st single dose)</td>
<td>42±4</td>
</tr>
<tr>
<td>Oxotremorine (cumulative)</td>
<td>22±4</td>
</tr>
<tr>
<td>Oxotremorine (2nd single dose)</td>
<td>32±3</td>
</tr>
<tr>
<td>Oxotremorine (cumulative--uncontrolled)</td>
<td>49±4</td>
</tr>
<tr>
<td>Acetylcholine (cumulative)</td>
<td>48±6</td>
</tr>
</tbody>
</table>

Maximum contractions in each experiment were obtained as follows: a single high dose (10 \mu{M}) of oxotremorine was used to contract the tissue (1st single dose), the drug was washed out, a cumulative oxotremorine concentration-response curve was obtained (cumulative), the oxotremorine washed out, and then a single high dose (10 \mu{M}) of oxotremorine was added (2nd single dose). In a separate experiment, the length of time between drug additions was only 1 to 2 minutes, which did not allow for a complete contraction before the next cumulative drug addition (cumulative--uncontrolled). Each value represents the mean ± S.E.M. for 4 tissues of each age group. Data for acetylcholine are from Figure 12.

* Significantly greater than 7 mo group (Duncan's, \( p < 0.05 \)).

** Significantly greater than both the 7 mo and 16 mo groups (Duncan's, \( p < 0.05 \)).

† Significantly less than 1st single dose \( E_{max} \) (t-test, \( p < 0.01 \)).
Figure 16. Cumulative concentration-response curves for oxotremorine-induced contractions of bladder neck tissues from 3 age groups.

The period of time between each successive drug addition was approximately 1 to 3 minutes which did not allow the tissue to fully contract before the next drug addition. Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as percentages of the maximum KCl-induced contraction for each tissue. No age-related changes in responsiveness were observed.
Figure 16

PERCENT OF KCL CONTRACTION

LOG MOLAR [OXOTREMORINE]
at rates that vary with age. That is, the 27 mo old tissues must
desensitize more rapidly than the 7 mo old tissues since the frequency
of drug addition to obtain Figure 16 (demonstrating no change in Emax)
was such that the 27 mo tissues were desensitized while the 7 mo tis-
sues had not begun to desensitize (Table 9).

Pilocarpine was nearly a full agonist in the bladder body and an
apparent partial agonist in the bladder neck (Table 6). The possibil-
ity that pilocarpine induced desensitization of the bladder neck was
also examined in an experiment identical to the oxotremorine experi-
ment above. The single concentration maximum contraction of pilocar-
pine was not significantly different from the Emax obtained by the
cumulative addition of pilocarpine to the tissues (Table 10). Hence,
pilocarpine does not appear to induce desensitization of the bladder
neck.

4.2.2 Binding studies.

A Scatchard analysis for the binding of \[^3H\]-QNB to the musca-
rinic receptors in the bladder tissues is depicted in Figure 17.
These plots are linear, consistent with the presence of a single class
of binding site in each tissue. Bmax and Kd determinations for
\[^3H\]-QNB binding in bladder necks and bladder bodies from rats of the
three age groups are shown in Table 11. In the bladder neck, an
18-30% increase in the Bmax (when expressed as fmol/mg tiss or fmol/
neck) was observed from 7 to 16 mo, while a 39-41% decrease was
observed from 16 mo to 27 mo. In the bladder body, a steady increase
in the Bmax (fmol/body) occurred with age, reaching a 108% when com-
paring 27 mo to 7 mo. However, the weight of this tissue also
### TABLE 10

**Effect of Age on Maximum Contractions Elicited by Pilocarpine in the Bladder Neck**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st single dose</td>
<td>24±4</td>
<td>32±5</td>
<td>29±10</td>
</tr>
<tr>
<td>Cumulative</td>
<td>16±4</td>
<td>26±4</td>
<td>24±2</td>
</tr>
<tr>
<td>2nd single dose</td>
<td>21±3</td>
<td>30±5</td>
<td>20±5</td>
</tr>
</tbody>
</table>

Maximum contractions in each experiment were obtained as follows: a single high dose (10 μM) of pilocarpine was used to contract the tissue, the drug was washed out, a cumulative pilocarpine concentration-response curve was obtained, the pilocarpine washed out, and then a single high dose (10 μM) of pilocarpine was added. Each value represents the mean ± S.E.M. for 4 tissues of each age group. No significant differences occurred between age groups or between values of maximum contractions for cumulative and single dose additions.
Figure 17. Saturation of $[^3H]$-QNB binding, plotted as bound/free vs. bound, in the bladder neck and body.

Age groups are designated as: 7 mo (▲), long dashes; 16 mo (■), short dashes; and 27 mo (●), solid line. Data shown are those from a single day's experiment with binding from each age group run in parallel.
### TABLE 11

Effects of Age on Bmax and Kd of \[^{3}H\]QNB Binding in the Bladder Neck and Body

<table>
<thead>
<tr>
<th>Tissue/Age</th>
<th>Kd (pM)</th>
<th>Bmax</th>
<th>Bmax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol/mg tiss</td>
<td>fmol/mg prot</td>
<td>fmol/region</td>
</tr>
<tr>
<td><strong>Body</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 mo</td>
<td>118±20</td>
<td>10.7±0.4</td>
<td>30.8±1.3</td>
</tr>
<tr>
<td>16 mo</td>
<td>126±13</td>
<td>11.5±0.9*</td>
<td>46.4±8.6</td>
</tr>
<tr>
<td>27 mo</td>
<td>110±13</td>
<td>13.4±0.9</td>
<td>49.4±8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neck</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 mo</td>
<td>95.4±14.2</td>
<td>6.99±0.85</td>
<td>55.0±4.0</td>
</tr>
<tr>
<td>16 mo</td>
<td>84.9±15.7</td>
<td>8.22±0.80</td>
<td>87.6±15.6</td>
</tr>
<tr>
<td>27 mo</td>
<td>94.0±21.0</td>
<td>4.98±0.57†</td>
<td>54.7±12.1</td>
</tr>
</tbody>
</table>

Saturation of \[^{3}H\]QNB binding was analyzed by linear regression of bound/free versus bound ligand (Scatchard, 1949). Each value represents the mean ± S.E.M. of four separate experiments for each age group.

* Significantly greater than 7 mo group (p < 0.05).

** Significantly greater than both the 7 mo and 16 mo groups (p < 0.05).

† Significantly less than 16 mo group (p < 0.05).
increases significantly with age (Table 5), and only a 25% increase in
Bmax occurs from 7 to 27 mo when expressed as fmol/mg tissue. Expression of Bmax values as fmol/mg protein showed no significant differences between ages, however similar trends in density changes are evident in both regions. The most consistent effect of age on the Bmax of $[^3H]$-QNB is a decrease observed in the bladder neck from ages 16 to 27 mo and an increase in the bladder body from 7 to 27 mo.

Differences in the densities of muscarinic receptors between the two regions also depend on the method used to normalize data. That is, the bladder neck has a higher density of receptors when Bmax is expressed as fmol/mg protein. However, if Bmax is expressed as fmol/mg tissue, the bladder body appears to have a higher density of receptors. This discrepancy is likely to be due to a difference in the amount of undetectable protein (possibly collagen) between the two regions when using the Lowry assay (see DISCUSSION). No significant regional difference in the affinity of $[^3H]$-QNB for muscarinic receptors occurred.

4.3 The Effects of Age on the Alpha Adrenoceptor System

4.3.1 Functional studies.

The purpose of these studies was to determine whether age affects the alpha adrenoceptor responsiveness of the bladder body and bladder neck. Furthermore, these studies were designed to elucidate the alpha adrenoceptor subtype(s) that mediate contraction in both regions of the bladder in young and senescent rats.

CR curves were obtained for the neurotransmitter norepinephrine, the selective alpha$_1$ adrenoceptor agonist phenylephrine, and the
selective alpha2 adrenoceptor agonist clonidine, in tissues from 7, 16, and 27 mo old rats (Figures 18-20). These curves were compared in terms of the Emax and ED50. Results for Emax of all drugs for both bladder regions of the three ages tested are shown in Figure 21 and Table 12. These results show a trend towards increasing Emax for all three agonists acting on the bladder body from aged animals. This increase reached statistical significance for phenylephrine and clonidine (p < 0.05). In contrast to the age-related changes in the bladder body, no changes in Emax were observed in the bladder neck.

Maximum contractions of the bladder body induced by alpha adrenoceptor agonists were also normalized to weight (Table 13). Using this method of normalization, a greater responsiveness to phenylephrine and clonidine was observed in the oldest group compared to the 2 younger groups, as was observed when the data was normalized to KCl contractions. However, this age effect was absent for norepinephrine.

Norepinephrine appeared to be a full agonist in the bladder neck but a partial agonist in the bladder body (Figure 21). Since the bladder body has a greater predominance of beta adrenoceptors than the bladder neck, it was conceivable that the 0.1 $\mu$M propranolol was not sufficient to fully antagonize the effects of norepinephrine at the detrusor beta adrenoceptors. In another experiment, 300 $\mu$M propranolol was used which resulted in a larger Emax for norepinephrine, but still well below the Emax of phenylephrine (Figure 22A, Table 14). In both the above experiments, propranolol was added 5 to 10 minutes prior to the cumulative norepinephrine CR curve, then both norepinephrine and propranolol were washed to allow tissue to reach resting tension ('intermittent' propranolol addition). In still another
Figure 18. Cumulative concentration-response curves for phenylephrine-induced contractions of bladder body and neck tissues from 3 age groups.

Tissues were preincubated with propranolol (0.1 μM) to antagonize the actions of phenylephrine at beta adrenoceptors. Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 18

PERCENT OF KCL CONTRACTION

LOG MOLAR [PHENYLEPHRINE]

BODY

NECK
Figure 19. Cumulative concentration-response curves for norepinephrine-induced contractions of bladder body and neck tissues from 3 age groups.

Tissues were preincubated with propranolol (0.1 μM) to antagonize the actions of norepinephrine at beta adrenoceptors. Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contraction are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 19
Figure 20. Cumulative concentration-response curves for clonidine-induced contractions of bladder body and neck tissues from 3 age groups.

Tissues were preincubated with propranolol (0.1 μM) to antagonize the actions of clonidine at beta adrenoceptors. Age groups are designated as 7 mo (▲); 16 mo (■); and 27 mo (●). Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentage of the maximum KCl-induced contraction for each tissue.
Figure 20
Figure 21. Maximum contractions (Emax) of bladder body and neck tissues of 3 age groups to phenylephrine (PE), norepinephrine (NE), and clonidine (CL).

All tissues were preincubated with propranolol (0.1 μM) to antagonize the actions of the agonists at beta adrenoceptors. Age groups are designated as: 7 mo (open) 16 mo (diagonal) and 27 mo (hatched). Each bar represents the mean ± S.E.M. of 4 to 6 tissues of each age group. Maximum contractions to alpha adrenoceptor agonists are expressed as a percentage of the maximum KCl-induced contraction of each tissue. * indicates significantly greater than 7 mo group. An age-related increase in the Emax values occurred in the bladder body, while no significant differences between age groups were observed in the bladder neck.
TABLE 12

Effects of Age on Maximum Contractions (Emax) Induced by Alpha Adrenoceptor Agonists in the Bladder Body and Neck

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Tissue</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine</td>
<td>body</td>
<td>49.4±7.7</td>
<td>55.6±5.0</td>
<td>78.4±3.4*</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>75.4±4.4</td>
<td>75.7±2.2</td>
<td>69.5±4.0</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>body</td>
<td>5.9±2.6</td>
<td>15.3±6.8</td>
<td>26.3±11.8</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>69.6±9.6</td>
<td>70.0±6.7</td>
<td>77.1±5.6</td>
</tr>
<tr>
<td>Clonidine</td>
<td>body</td>
<td>11.4±3.6</td>
<td>11.9±5.9</td>
<td>31.3±4.1*</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>20.1±1.9</td>
<td>24.8±3.2</td>
<td>19.5±2.5</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of the Emax values from 4 to 6 tissues per age group. Emax values for phenylephrine and clonidine were obtained in the same bladder tissues. All tissues were incubated in the presence of 0.1 μM propranolol.

* Significantly greater than 7 mo group (p < 0.05).
TABLE 13

Maximum Contractions (E_{max}) Induced by Alpha Adrenoceptor Agonists Normalized to Bladder Body Weight

<table>
<thead>
<tr>
<th>Agonist</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine</td>
<td>105±19</td>
<td>85±18</td>
<td>132±8</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>122±22</td>
<td>130±7</td>
<td>121±11</td>
</tr>
<tr>
<td>Clonidine</td>
<td>25±8</td>
<td>21±13</td>
<td>53±8</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of the E_{max} values from 4 to 6 tissues per age group. E_{max} values for phenylephrine and clonidine were obtained in the same bladder tissues. All tissues were incubated in the presence of propranolol at the following concentrations: 0.1 μM for phenylephrine and clonidine; 100 μM for norepinephrine. E_{max} values for norepinephrine were calculated based on individual bladder body weights while E_{max} values for phenylephrine and clonidine were computed using a mean bladder body weight of the 6 tissues used.

* Significantly greater than 16 mo group (p < 0.05).
Figure 22. Cumulative concentration-response curves for norepinephrine-induced contractions of bladder body tissues preincubated with 300 μM propranolol intermittently (A) and 100 μM propranolol continuously (B).

Age groups are designated as ▲, 7 mo; ■, 16 mo; and ●, 27 mo. Each point represents the mean ± S.E.M. for 4 tissues in each group. Contractions are expressed as the percentages of the maximum KCl-induced contraction for each tissue.
Figure 22
TABLE 14

Effect of Propranolol Concentration and Incubation Conditions on Maximum Norepinephrine-induced Contractions of the Bladder Body

<table>
<thead>
<tr>
<th>Propranolol Concentration</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-7}$ M$^a$</td>
<td>5.9±2.9</td>
<td>15.3±6.2</td>
<td>26.3±8.6</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$ M$^b$</td>
<td>63.0±5.6</td>
<td>77.0±6.8</td>
<td>79.2±5.6</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$ M$^a$</td>
<td>25.7±3.3</td>
<td>37.6±3.3</td>
<td>35.3±4.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of the Emax values from 4 bladder body tissues from each age group. Although a trend towards age-related increase in responsiveness is evident, there are no statistical differences between age groups.

$^a$ Propranolol was added to the bath 5 to 10 minutes prior to obtaining the norepinephrine concentration response curves.

$^b$ Bladder body tissues were continuously incubated with propranolol.
experiment with norepinephrine, the bladder body tissues were continuously incubated with 100 µM propranolol, including during the 45 minute equilibration period prior to obtaining CR data. This method resulted in Emax values for norepinephrine equivalent to those of phenylephrine (Figure 22B, page 112; Table 14, page 114); however, the magnitude of the age-related increase in responsiveness of the body was reduced from a 333% increase (0.1 µM propranolol) to a 25% increase (100 µM propranolol).

The ED50 values (expressed as pD₂) for all drugs acting on both regions of the bladders of the three age groups are shown in Table 15. No significant age-related changes were observed in the ED50 for any of the drugs acting on either of the bladder regions.

Norepinephrine was 10- to 100-fold less potent in mediating bladder contractions than reported for its actions on other tissues, e.g. the rat aorta (Owen and Carrier, 1980) or the rabbit uterus (Hoffman et al., 1981). One possible explanation is that uptake mechanisms were not blocked in these studies. Therefore, experiments were designed to determine how much influence norepinephrine uptake into nerve terminals has in the rat urinary bladder. Blocking uptake in tissues from each age group also permitted the removal of any influence that the aged uptake process might have on the CR data observed previously. Treatment of the bladder neck from 7, 16 and 27 mo rats with cocaine (100 µM) and hydrocortisone (30 µM) shifted the CR curves to norepinephrine to the left in the bladder neck (Table 16). This shift resulted in significant decreases in the ED50 values of norepinephrine in necks from all 3 age groups (p < 0.05).
TABLE 15

pD₂ Values of Alpha Adrenoceptor Agonists in the Bladder
Body and Neck from Rats of 3 Age Groups

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Tissue</th>
<th>7 mo</th>
<th>16 mo</th>
<th>27 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine</td>
<td>body</td>
<td>5.17±0.09</td>
<td>5.13±0.10</td>
<td>5.40±0.08</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>4.82±0.07</td>
<td>4.89±0.12</td>
<td>4.99±0.12</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>body</td>
<td>4.73±0.09</td>
<td>4.92±0.03</td>
<td>4.92±0.05</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>4.71±0.07</td>
<td>4.83±0.04</td>
<td>4.92±0.02</td>
</tr>
<tr>
<td>Clonidine</td>
<td>body</td>
<td>5.04±0.39</td>
<td>5.28±1.10</td>
<td>5.54±0.37</td>
</tr>
<tr>
<td></td>
<td>neck</td>
<td>6.36±0.07</td>
<td>6.25±0.07</td>
<td>6.29±0.05</td>
</tr>
</tbody>
</table>

Each pD₂ value (pD₂ = -log ED50) represents the mean ± S.E.M. for
4 to 6 determinations in each age group. pD₂ values for phenyl-
ephrine and clonidine were obtained in the same bladder tissues.
All tissues were incubated in the presence of propranolol at the
following concentrations: 0.1 µM for phenylephrine and clonidine;
100 µM for norepinephrine. No significant differences between age
groups were observed, judged by the 95% C.I. of the means.
### TABLE 16

Effect of Cocaine and Hydrocortisone Treatment on pD\textsubscript{2} Values of Norepinephrine in Bladder Tissues from Rats of 3 Age Groups

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Tissue</th>
<th>Control (pD\textsubscript{2})</th>
<th>Hydrocortisone + Cocaine (pD\textsubscript{2})</th>
<th>Magnitude of Shift\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>7mo</td>
<td>bladder neck</td>
<td>4.71±0.07</td>
<td>5.34±0.08 *</td>
<td>4.27</td>
</tr>
<tr>
<td>16mo</td>
<td>&quot;</td>
<td>4.83±0.04</td>
<td>5.59±0.04 *</td>
<td>5.75</td>
</tr>
<tr>
<td>27mo</td>
<td>&quot;</td>
<td>4.92±0.02</td>
<td>5.50±0.07 *</td>
<td>3.80</td>
</tr>
<tr>
<td>7mo</td>
<td>bladder body</td>
<td>4.73±0.09</td>
<td>4.69±0.11</td>
<td>0.91</td>
</tr>
<tr>
<td>16mo</td>
<td>&quot;</td>
<td>4.92±0.03</td>
<td>4.91±0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>27mo</td>
<td>&quot;</td>
<td>4.92±0.05</td>
<td>4.93±0.04</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Data presented are means ± S.E.M. of pD\textsubscript{2} values from four bladder tissues per age group (pD\textsubscript{2} = -log ED50). Concentration response curves were obtained in the presence and absence of 100\,\mu M cocaine plus 30\,\mu M hydrocortisone. All concentration response curves were obtained in the presence of propranolol (0.1\,\mu M, neck; 0.3\,\mu M body).

\textsuperscript{a} Magnitude of shift was the ED50 of the treated tissue divided by the ED50 of the control.

* Significantly different from the pD\textsubscript{2} of control for that age group, as judged by the lack of overlap of the 95% C.I. of the means.
Furthermore, the magnitude of the this shift remained relatively constant with age at a 3.8- to 5.8-fold increase in potency. In the bladder body, cocaine and hydrocortisone treatment did not affect the ED50 values of norepinephrine in any age group. In all age groups, the maximum contractions induced by norepinephrine in both bladder regions were unchanged after treatment with cocaine and hydrocortisone.

The age-related increase in Emax for phenylephrine and clonidine could result from parallel changes in action at both alpha_1 and alpha_2 adrenoceptors or from both drugs acting on the same receptor. These possibilities were examined by exposing the tissues to clonidine prior to the addition of phenylephrine. Clonidine (100 μM) produced a contraction 1/5 that of phenylephrine (100 μM) alone in the bladder body and 1/3 that of phenylephrine (100 μM) alone in the bladder neck. Clonidine (100 μM) completely inhibited any further contraction induced by phenylephrine (100 μM) in the bladder body and the bladder neck from both 7 mo rats (Figure 23) and 27 mo rats. Thus, clonidine acts as a partial agonist at the phenylephrine receptor in both young and senescent tissues from both bladder regions.

Prior to these studies, the alpha adrenoceptor subtype responsible for contraction of the two bladder regions had not been classified. Hence, experiments were designed to subclassify the alpha adrenoceptors of the two bladder regions and also to determine which subclassification was responsible for the age-related increase in alpha adrenoceptor responsiveness of the bladder body. To accomplish this, the pA₂ values of the selective alpha_1 antagonist prazosin
Figure 23. Antagonism of phenylephrine (PE) induced contractions by clonidine (Cl) in the bladder body and neck from 7 mo rats.

Tissues were preincubated with propranolol (Prop) to antagonize effects of the alpha agonists at beta adrenoceptors. Clonidine completely antagonized phenylephrine induced contractions indicating that clonidine is a partial agonist, while phenylephrine is a full agonist at the same receptor. Phenylephrine induced contractions were antagonized by prazosin (Praz) indicating that the receptor subtype is alpha$_1$. 
Figure 23
(pA₂ = 8.5 to 9.0 for an alpha₁ antagonism) and the selective alpha₂ antagonist yohimbine (pA₂ = 7.5 to 8.0 for an alpha₂ antagonism) were examined using the agonist phenylephrine to contract bladder body and neck tissues from 7 and 27 mo old rats. Schild plots of the data for prazosin and yohimbine (Figure 24, body; Figure 25, neck) were drawn and the pA₂ values (abscissa intercepts) and slopes appear in Table 17. The Kᵦ values (pA₂ = -log Kᵦ) of yohimbine were 300-390 times greater than those of prazosin in 7 and 27 mo old bladder body tissues, and 130-160 times greater than those of prazosin in 7 and 27 mo old bladder neck tissues. The slopes of the Schild plots were near unity in all cases. Thus, based on the much greater affinity of prazosin than yohimbine for the adrenoceptors of both bladder regions, it is concluded that the alpha adrenoceptor subtype mediating contractions in both bladder regions is alpha₁. Since there was no age-related changes in the pA₂ values of either antagonist with age, the receptor subtype mediating contractions in the aged bladder body is also alpha₁.
Figure 24. Schild plot demonstrating the relative antagonism of phenylephrine-induced contractions by the alpha_1 antagonist, prazosin (solid lines) and the alpha_2 antagonist, yohimbine (broken lines) in the bladder body. Age groups are designated as ▲, 7 mo and ●, 27 mo. Each point represents the mean ± S.E.M. of data from 3 tissues. The higher affinity of prazosin compared to yohimbine indicates that phenylephrine-induced contractions of the body are mediated via alpha_1 adrenoceptors.
Figure 25. Schild plot demonstrating the relative antagonism of phenylephrine-induced contractions by the alpha₁ antagonist, prazosin (solid lines) and the alpha₂ antagonist, yohimbine (broken lines) in the bladder neck. Age groups are designated as ▲, 7 mo and ●, 27 mo. Each point represents the mean ± S.E.M. of data from 3 tissues. The higher affinity of prazosin compared to yohimbine indicates that phenylephrine-induced contractions of the neck are mediated via alpha₁ adrenoceptors.
Figure 25

LOG (DOSE RATIO - 1) vs LOG MOLAR [ANTAGONIST]

NECK
<table>
<thead>
<tr>
<th></th>
<th>Prazosin</th>
<th></th>
<th>Yohimbine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pA_2)</td>
<td>slope</td>
<td>(pA_2)</td>
</tr>
<tr>
<td><strong>Body</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 mo</td>
<td>8.57±0.28</td>
<td>1.20</td>
<td>5.98±0.12</td>
</tr>
<tr>
<td>27 mo</td>
<td>8.66±0.13</td>
<td>1.16</td>
<td>6.18±0.23</td>
</tr>
<tr>
<td><strong>Neck</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 mo</td>
<td>8.85±0.10</td>
<td>1.17</td>
<td>6.74±0.16</td>
</tr>
<tr>
<td>27 mo</td>
<td>8.85±0.12</td>
<td>1.04</td>
<td>6.64±0.04</td>
</tr>
</tbody>
</table>

Each \(pA_2\) value represents the mean ± S.E.M. for 3 separate \(pA_2\) determinations made in 3 tissues of each age group. The slope values are the slopes of the regression lines of the combined data for each antagonist.
CHAPTER V

DISCUSSION

5.0 Overview

Examination of urinary bladder function in healthy elderly individuals by Broklehurst and Dillane (1966), Broklehurst (1979) and Andersen (1978) revealed several alterations: decreased bladder capacity, residual urine, uninhibited detrusor contractions, sensory deficits, slow urine flow, and bladder neck funneling. Since the activity of the bladder is under the direct influence of both the sympathetic and parasympathetic nervous systems, it seems likely that with age, an upset of the autonomic control of the bladder could contribute to bladder dysfunction in the elderly.

Using the Fischer 344 (F344) rat, Kolta et al. (1984) demonstrated an enhanced responsiveness of the aged bladder to acetylcholine. This report was the first experimental demonstration of change in the autonomic responsiveness of the aged bladder. The present studies were designed to extend this finding—to determine whether the autonomic neuroreceptor systems of the two anatomically, functionally and pharmacologically distinct regions of the bladder are affected equally by age and to determine whether alterations in receptor populations or affinities could explain any observed functional changes.
Briefly, the results of the present study demonstrate that age-induced changes in both cholinergic muscarinic and alpha adrenergic receptor systems of the bladder occur; however, these changes are regionally specific. Furthermore, at least for the muscarinic receptor system, functional change does not correlate with change in the number of receptors determined by radiolabelled antagonist binding. The discussion of these findings will consider first the appropriateness of the aging animal model selected, then changes in the cholinergic receptor system followed by discussion of alterations in the alpha adrenoceptor system. Finally, the effect that these regional changes may have on the bladder as a single functioning unit will be discussed.

5.1 The F344 Rat Urinary Bladder as a Model for Aging Research.

A major goal of the present research was to investigate the possibility that age-related alterations occur in the responsiveness of the urinary bladder to autonomic agents using the isolated F344 rat urinary bladder. This animal model was selected for a number of reasons. First, selection of an animal for aging research depends heavily upon the cost and availability of aged animals. Certainly, use of the cat or rabbit bladder would be less tedious than the rat bladder simply because of the mere size of the organ. However, the life spans of the cat and rabbit are between 8 and 15 years, causing it to be costly to rear these animals to old age, and consequently, their availability is limited. In this regard, the aged rat is much less expensive, having an average life span of about 2.5 years. Second,
similarity of the neuropharmacology of the animal bladder to that of
the human bladder is necessary in order to predict similar age
effects. As shown in Table 18, the isolated rat and human bladder
respond very similarly to autonomic agents. Furthermore, the rat has
been used as an animal model of aging in many different biological
investigations, and many similarities in the effects of age between
the rat and man are known to exist (Committee on Animal Models for
Research on Aging, 1981; Roth and Hess, 1982).

Within the rat species, strains most often selected for aging
research are Sprague-Dawley, Wistar, Long Evans and Fischer. Pharma­
cological investigations of age predominantly utilize the Fischer and
Wistar rat strains. Although the F344 rat was used in the present
research, similar findings of age effects in bladders from other
strains or species would be supportive of a generalization of these
effects to human bladder aging.

5.2 The Effect of Age on Cholinergic Muscarinic Responsiveness

5.2.1 Overview of findings.

The age-related increase in the maximum cholinergic agonist-in­
duced contractions of the urinary bladder was regionally specific,
occuring only in the bladder neck and completely absent in the blad­
der body. These regionally specific changes were consistent for all
cholinergic agonists tested. Of these agonists, acetylcholine and
pilocarpine activate both nicotinic and muscarinic receptors, while
both betahanechol and oxotremorine preferentially activate muscarinic
receptors. Since the increased responsiveness in the bladder neck was
## TABLE 18

Responses of the Rat and Human Urinary Bladders to Autonomic Effectors

<table>
<thead>
<tr>
<th>Effector</th>
<th>Rat</th>
<th>Human</th>
<th>Effector</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha adrenoceptor agonist in presence of propranolol</td>
<td>+</td>
<td>+</td>
<td>Alpha adrenoceptor agonist in presence of propranolol</td>
<td>a</td>
<td>a,b</td>
</tr>
<tr>
<td>Beta adrenoceptor agonist in presence of phentolamine</td>
<td>-</td>
<td>-</td>
<td>Beta adrenoceptor agonist in presence of phentolamine</td>
<td>a</td>
<td>a,b</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>+</td>
<td>+</td>
<td>Acetylcholine</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Nerve stimulation</td>
<td>+ e</td>
<td>?</td>
<td>Nerve stimulation</td>
<td>f</td>
<td>?</td>
</tr>
<tr>
<td>Atropine inhibition of nerve-stimulated contraction</td>
<td>60% e</td>
<td>?</td>
<td>Atropine inhibition of nerve-stimulated contraction</td>
<td>100% f</td>
<td>?</td>
</tr>
</tbody>
</table>

+ refers to contraction; - refers to relaxation

<table>
<thead>
<tr>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Awad et al., 1974.</td>
</tr>
<tr>
<td>b Benson et al., 1976.</td>
</tr>
<tr>
<td>c Ek et al., 1977.</td>
</tr>
<tr>
<td>d Nergardh, 1975.</td>
</tr>
<tr>
<td>e Alm and Elmer, 1975.</td>
</tr>
</tbody>
</table>
observed for all four cholinergic agonists, the receptors mediating the enhanced contractions in the aged tissues are most likely muscarinic. It was anticipated that an age-related increase in the number of muscarinic receptors in the bladder neck might mediate the observed change in responsiveness; however, a paradoxical decrease in the muscarinic receptor density as determined by $[^3H]$-QNB binding was observed. Moreover, the largest increase in functional muscarinic responsiveness occurred between the ages of 16 and 27 mo, when the largest decrease in muscarinic receptor density occurred (Figure 26). Hence, the age-related increase in muscarinic responsiveness of the bladder neck cannot be easily explained by a change in the number of muscarinic receptors in this tissue.

5.2.2 Normalization of response to muscarinic receptor activation.

The normalization of data by expressing drug-induced contraction as a percentage of the maximum KCl-induced contraction, or by expressing contractions in mm tissue movement per g wet tissue weight did not affect the magnitude of the age-related change in the responsiveness of the bladder neck. This is not surprising in a tissue which demonstrates no age-dependent weight change and no age-dependent change in responsiveness to KCl. However, the bladder body does increase in weight with age, and when the Emax values of the agonists are normalized to weight, a 12 to 25% decrease in the Emax is apparent for bethanechol and oxotremorine; however, these changes are not statistically significant. These findings indicate that it may be enlightening to examine the muscarinic agonist-induced contraction of the bladder body isometrically. Isometric contractions are a measure of the
Figure 26. The relationship between changes in contractility and number of muscarinic receptors in the bladder neck with age.

Data are expressed as the percentage change from data of the 7 mo age group. The solid line depicts the percentage change in the Emax of bethanechol. The broken line depicts the percentage change in the number (Bmax, fmol/mg tiss) of muscarinic receptors measured by [3H]-QNB binding.
Figure 26
force produced by a unit weight of muscle tissue and, therefore, would provide information on any effects of age on the ability of the bladder body to isometrically contract against a volume of urine.

5.2.3 Postulated explanations of the lack of correlation between functional and binding experiments.

Changes in the physiological response of a tissue to agonists are not always explained by a change in the density of receptors, although many such instances occur (see Roth and Hess, 1982 for review). In the heart, a 43% decrease in cholinergic responsiveness with age (Kelliher and Conahan, 1980) cannot be explained by a similar decrease in the Bmax of $[^3H]$-QNB binding, and in fact, an approximate 25% increase in Bmax with age was observed in the atria with no change in the ventricles (Narayanan and Derby, 1983). In contrast, in the present study the opposite reciprocal relationship was observed. In the bladder neck between the ages of 16 and 27 mo, a 36% increase in cholinergic responsiveness was accompanied by a 39% decrease in muscarinic receptors. The inverse relationship between function and receptor number may indicate that changes in responsiveness result from alterations in the influence of substances which modulate the binding of muscarinic agonists to their receptors (GTP, Na$^+$, Mg$^{2+}$) or result from post-receptor alterations, such as a change in the coupling of the activated receptor to contractile elements. Furthermore, these changes may imply that antagonists and agonists bind to different sites associated with the muscarinic receptor which may differ in number; however, no evidence for separate agonist and antagonist sites exists for the muscarinic receptor.
One shortcoming of correlating binding data to functional data is that radiolabelled antagonists (e.g. $[^3\text{H}]\text{QNB}$) used typically to determine receptor densities, do not identify coupled receptors or receptors capable of coupling, but instead label all receptors of a given receptor class. It is quite possible that receptors coupled to an effect (contraction) are different in number from the total number of receptors; in fact, in many tissues spare receptors exist (Ruffolo, 1982). If spare receptors exist in the bladder neck, which is likely, then with age some spare receptors could be lost while others could become coupled to response. However, spare receptors determine the position of the CR curve along the log concentration axis and thus, determine the ED50 (Ruffolo, 1982). Hence, in opposition to the possibility that a change occurs in the spare receptor population of the aged bladder neck is the lack of an age-related change in the ED50 values of muscarinic agonists.

Coupling of muscarinic receptors to functional response in cardiac muscle has been demonstrated to be regulated by guanine nucleotides (Halvorsen and Nathanson, 1984). Whether bladder muscarinic receptors are similarly regulated by guanine nucleotides is not known presently; however, an effect of age on such regulation resulting in enhanced coupling of muscarinic receptors to contraction of the bladder neck could underly the observed changes. Whatever the explanation for the lack of correlation, observations of inversely related functional and receptor density changes with age indicate that, in cases where changes in receptor density parallel changes in function, it may be erroneous to assume that the change in receptor density is a
causative factor for a change in function. Likewise, it may be erro-
neous to assume that the functional responsiveness of a tissue is not
altered when it is observed that the density of receptors does not
change. Hence, receptor binding techniques should be used with a
great deal of caution when searching for conclusions regarding changes
in the response of a tissue since the existence of a receptor does not
necessarily imply that that receptor is functional.

5.2.4 Other receptor binding considerations.

The density of muscarinic receptors in the bladder body increases
with age while that of the neck decreases (Table 11). In the whole
rat bladder, an increase (Kolta et al., 1984) and no change (Hayes et
al., 1983) in $[^3H]$-QNB binding have been reported. Strain differences
in the effects of age on muscarinic receptor density may exist, since
the Kolta study used F344 rats while the Hayes study used Sprague-Daw-
ley rats. However, the disparity in the findings of the two whole
bladder studies could be a result of different isolation techniques,
e.g. excision of mostly the body region (where age increases receptor
density), or excision of the entire bladder including the neck region
(where age decreases receptor density). In examining the muscarinic
receptor density in the whole bladder, an increase in the bladder body
could mask a decrease in the neck since the neck comprises only 25 to
33% of the entire bladder weight. Kolta et al. (1984) also demon-
strated an increase in muscarinic responsiveness in the whole bladder,
while the present study demonstrates that the increase occurs only in
the bladder neck. Hence, in the bladder, where two general regions
subserve different physiological functions, conclusions based on
functional and biochemical assessments of the whole tissue must be carefully considered.

$E_{\text{max}}$ values of $[^3\text{H}]\text{QNB}$ were reported as fmol/mg tissue, fmol/mg protein and fmol/whole tissue (Table 11); interpretation of the results depends on which method of data expression is chosen. The expression of densities as fmol/mg protein in aging bladder research as is often done (Kolta et al., 1984; Hayes et al., 1983) may be misleading in these tissues for the following reasons. The wet weight of the bladder neck does not change substantially with age, while the percentage protein undergoes a rather marked 33% decrease from 7 to 27 mo (see Table 5). The decrease in percentage protein does not reflect a general decrease in solid bladder components as no change in the dry weight of this tissue with age has been observed. The change in percentage protein may be artifactual, i.e. resulting from only a change in the amount of detectable protein. Detectable protein in the Lowry assay, as in other assays, is typically that amount of protein which dissolves in 0.1 N NaOH. Since homogenates of bladder tissue are incompletely dissolved, even under rigorous conditions (see METHODS), measured protein might not reflect total protein if there are age-dependent changes in solubility. The urinary bladder contains approximately 25% collagen (Swaiman and Bradley, 1967) which increases with age (Susset et al., 1978). Furthermore, collagen stability has been shown to increase with age (Sobel, 1968). This increase in the stability of connective tissue protein could render certain protein of the aged bladder undetectable by the Lowry assay, and furthermore, may explain some of the discrepancies between binding data expressed as
fmol/mg tissue weight and fmol/mg protein (see Table 11). Hence, the most reliable estimations of receptor density in these studies are fmol/whole tissue and fmol/mg tissue.

5.3 The Effect of Age on Alpha Adrenoceptor Responsiveness

5.3.1 Overview of findings.

Age-related change in the alpha adrenoceptor responsiveness of the bladder was also regionally specific. Age-related increases in Emax values of alpha adrenoceptor agonists in the bladder body were observed with age, while no changes were apparent in the bladder neck. This, of course, is in sharp contrast to the regionally specific change in the muscarinic responsiveness of the bladder neck. Furthermore, age-related increases in the responsiveness of the bladder body were consistent for the three alpha adrenoceptor agonists examined. Determination of pA2 values of the selective alpha1 and selective alpha2 antagonists, prazosin and yohimbine, respectively, in bladder regions of the three age groups demonstrated that the receptor subtype responsible for the increased responsiveness of the bladder body is alpha1 (Table 17). Attempts to determine receptor density using radiolabelled antagonists were unsuccessful. Problems confronted in these attempts and their possible causes are discussed in Section 3.3.

5.3.2 Age-related change in alpha adrenoceptor responsiveness.

An age-related increase in the responsiveness of the bladder body was consistent for phenylephrine, norepinephrine and clonidine. These age effects were apparent when the Emax values were expressed as percents of the maximum KCl-induced contractions. However, age-related
increases in the Emax values were less marked when data were normalized to the weight of the bladder body. As mentioned in Section 5.1.2, the maximum contractions induced by KCl (expressed as mm tissue movement) do not change with age. However, the magnitude of these contractions appear to decrease with age when normalized to bladder body weight since the weight of the bladder body increases significantly with age. This decrease does not reach statistical significance, but raises some important questions. Normalization of CR data by expressing drug-induced contractions as percentages of the maximum contractions induced by KCl is often used in the aging literature (Yanagawa et al., 1978; Scott, 1982). A disadvantage of this method is that if age alters the responsiveness of a tissue to KCl, then drug responsiveness will be obscured when expressed as percentages of the age-altered KCl response. Other methods which have been used to normalize data often involve the weight of the tissue (Simpkins et al., 1983; Tuttle, 1966). However, the normalization to KCl-induced contractions is favored for a number of reasons. First, the variability of tissue length within a given age group is eliminated. Second, we have observed an approximate 40 to 50% increase in the weight of the bladder body with age. Since smooth muscle tissue is post-mitotic, an increase in bladder weight with age may correspond to an increase in the size of the smooth muscle cells but may also result from increases in any number of non-contractile tissues or elements which comprise the bladder, e.g. epithelial, adipose, collagen, elastin, vascular. As mentioned in Section 5.1.2, there is an age-related increase in human bladder collagen content (Susset et al., 1978).
Furthermore, although data for the urinary bladder are lacking, in liver, kidney, and vascular tissue of the aged rat the percent increase in collagen content is greater than the percent increase in the total organ weight (Schaub, 1963). Thus, normalization of data to KCl contractions seems most reliable as this parameter does not change with age and it relates directly to the contractile elements of the tissue. Hence, it can be concluded that while no changes were observed in the bladder neck, there was an age-related increase in the responsiveness of the bladder body to alpha adrenoceptor agonists.

5.3.3 Alpha adrenoceptor subtype mediating contraction.

Several lines of evidence indicate that the alpha₁ adrenoceptor subtype is involved in the age-related increase in responsiveness of the bladder body. 1) Phenylephrine, which is selective for alpha₁ adrenoceptors, produced a relatively large Emax (Figure 21). 2) The -log ED₅₀ of clonidine was about 6 in the bladder neck and 5.5 in the bladder body of all three age groups (Table 15). These values are characteristic of alpha₁ adrenoceptor interactions since the -log ED₅₀ for clonidine at alpha₁ adrenoceptors in other tissues is 5 to 6, while the -log ED₅₀ for clonidine at alpha₂ adrenoceptors is 7.5 to 8.5 (Ruffolo et al, 1980). 3) Clonidine completely antagonized any further contraction induced by the full agonist, phenylephrine, in both regions of the bladder, whether from bladders of 7 mo or 27 mo old rats, providing support for a homogeneous population of alpha adrenoceptors. 4) Finally, the pA₂ values of the selective alpha₁ antagonist, prazosin and the selective alpha₂ antagonist, yohimbine showed that prazosin has a much greater affinity for the adrenoceptors
in both bladder regions of both 7 and 27 mo rats (Table 17). Hence, it is concluded that the alpha adrenoceptor subtype mediating contractions in both bladder regions is alpha_1, and this receptor subtype mediates the age-related increase in bladder body responsiveness to alpha adrenoceptor agonists.

Differences in the properties of the alpha adrenoceptors of the two bladder regions have been reported. For example, Downie et al. (1975) demonstrated a fourfold to fivefold higher affinity of norepinephrine in the bladder neck than in the bladder body of the rabbit. Furthermore, Barker et al. (1977) reported that the isomeric activity ratios of norepinephrine were significantly different in the two bladder regions of rabbits. Our observations also indicate differences between the effects of the adrenergic agonists in the two regions of the rat bladder. For example, the ED50 of clonidine was tenfold lower in the bladder neck than in the bladder body. Furthermore, the affinity of yohimbine was fivefold greater in the neck than the body. The higher affinity of these two alpha_2 selective compounds indicate that alpha_1 and alpha_2 adrenoceptors may coexist in the bladder neck. Disparities between the two regions in the action of these substances may also be due to differences in the ability of the agonist or antagonist to reach the receptor. For example, as mentioned in Section 1.1.2, the adrenergic nerve terminals of the two regions may have different ganglionic origins and hence, may be structured differently enough to cause a disparity between the regions in the availability of the receptor to exogenous agonist.

Another difference between the two regions was that after treatment of the tissues with the norepinephrine uptake blockers, cocaine
and hydrocortisone, the CR curves to norepinephrine in the bladder neck were shifted significantly to the left (approximately fourfold), whereas the CR curves to norepinephrine in the bladder body were unaffected (Table 16). One would expect uptake to have a greater effect in a tissue which is more densely innervated. Thus, these results are in agreement with theoretical expectations since the bladder neck of the rat has a much greater density of adrenergic nerves than does the bladder body (El-Badawi and Schenk, 1966).

5.3.4 Theoretical considerations of altered alpha adrenoceptor responsiveness.

The increase in the alpha-adrenergic responsiveness of the bladder body may be mediated by a change in the number of alpha adrenoceptors, changes in the coupling behavior of the alpha adrenoceptor, and/or changes in post-receptor biochemical mechanisms. The possibility of an increase in the number of alpha adrenoceptors in the bladder body seems most attractive. This alteration would upset the balance of alpha and beta adrenoceptors in the bladder. Such an alteration in the balance of adrenergic organization has been observed experimentally in the bladder of the cat. Autonomic denervation of the feline bladder results in an increase in the density of adrenergic terminals and a change in the type of preferentially activated receptors, from beta to alpha adrenoceptors, in the bladder body with receptor function and terminal density remaining unchanged in the bladder neck (Dahlström, 1978). A decline in autonomic activity may have an effect on bladder innervation similar to autonomic denervation. A decline with age in the activity of the autonomic control of other organs has
been reported (Frolkis et al., 1970; Thompson et al., 1974; Collins et al., 1980; Kelliher and Conahan, 1980). Therefore, if a decline in activity of the autonomic nervous system occurs with age in the F344 rat, the result may be an adaptive change in bladder innervation by which sympathetic nerve terminals and accompanying alpha adrenoceptors appear in the bladder body. Unfortunately, my attempts to examine alpha adrenoceptor number have been unsuccessful, possibly because of a very low density and therefore undetectable number of receptors. Even if an increase in the number of alpha adrenoceptors had been demonstrated, the question of whether the new receptors were functional or spare would need to be answered (see 5.1.3). Hence, other markers for enhanced alpha adrenoceptor responsiveness (Lefkowitz and Hoffman, 1980; Exton, 1982) would be needed (e.g. enhanced phosphatidylinositol turnover, enhanced receptor-mediated rise in intracellular calcium concentration) along with evidence of an increase in adrenergic terminal density (e.g. increase in levels of norepinephrine monoamine oxidase or dopa-decarboxylase) to support this hypothesis.

5.4 Potential Effects of Observed Changes in the Muscarinic and Alpha Adrenergic Receptor Systems on Bladder Function

During the filling phase of bladder function, the bladder body maintains a degree of muscle tone which accounts for the bladder pressure of 10 cm H₂O in the human (see section 1.1.4); likewise muscle tone of the bladder outlet and urethra region maintains a tonic contraction to resist the loss of urine. During the expulsion phase the bladder body contracts and forces the urine through the urethra. This contraction results in shortening of the smooth muscle fibers of the
bladder body to the point of eliminating intravesicular space. Furthermore, the longitudinal muscles of the bladder neck shorten and pull open the bladder outlet, facilitating the expulsion of urine. Thus, the bladder is both an isometric (storage) and isotonic (expulsion) muscle.

Age-related changes in the isotonic contractions of the bladder regions induced by autonomic agents were observed in the present study. However, changes in isotonic contractions may also imply that similar changes exist in isometric contractions. An enhanced alpha adrenergic responsiveness of the bladder body would upset the normal predominance of beta adrenoceptor function in this region, possibly resulting in an increased tone of the bladder body during filling. This increased tone could result in greater bladder filling pressure and would likely reduce bladder capacity. Enhanced muscarinic responsiveness of the bladder neck with age may also reflect an increase in the tone of this region during bladder filling, indicating a shortening and funneling of the bladder neck region which could decrease intraurethral pressure and result in loss of urine.

Definitive conclusions concerning the relationship between changes in the contractility of the aged isolated bladder and the development of geriatric urinary bladder dysfunction are certainly premature at this time. If the age-related changes observed in the rat urinary bladder can be extrapolated to humans, they may explain some of the observed abnormalities of the aged human bladder. In terms of the isolated bladder neck, an increase in muscarinic responsiveness occurs with age and might reflect an increase in the tone of
the smooth muscles in this region, since even at low concentrations of agonist an age-related enhanced contraction is evident. An increase in bladder neck tone may contribute to the funneling of the bladder neck observed by Brocklehurst (1979) in aged human bladder.

An increase in the alpha-adrenergic responsiveness of the bladder body may be part of the pathophysiological basis of unstable detrusor contractions observed in a high percentage of elderly continent individuals by Brocklehurst (1966) and Andersen (1978). That is, norepinephrine released from adrenergic neurons in the normal bladder during the filling phase activates beta adrenoceptors which mediate relaxation of the body. Age may change the dominant adrenergic receptor in the bladder body from beta to alpha. This alteration would result in contractions of the body when norepinephrine is released from adrenergic neurons during filling. These contractions, if strong enough, could conceivably result in spillage of urine during filling and storage of urine. In support of a role for bladder body alpha adrenoceptors in the development of incontinence are the findings of Jenson (1981) demonstrating an increase in the alpha-adrenergic activity of the bladder in patients suffering from incontinence due to neurogenic bladder disease. Furthermore, in detrusor strips from patients suffering from incontinence due to lower motor neuron lesions, an increase in the number of adrenergic fibers as well as alpha adrenoceptor function has been reported (Sundin et al., 1977). Incontinence in these disorders, and possibly in the aged, may be partly a result of a relative increase in the alpha adrenoceptor mediated contractility of the bladder body over that of the bladder neck, a condition we
observed in the aged rat. These possible alterations are clearly speculative at the present time. Regionally specific changes in the muscarinic and alpha adrenergic receptor systems do occur in the bladder and these findings are only the beginning in our understanding of the underlying neuropathology of urinary bladder dysfunction in the elderly.

5.4 Future Research Directions

This research has served to characterize the effects of age on the autonomic responsiveness of the rat urinary bladder, and lends support to the hypothesis that age-related changes in the autonomic control of the urinary bladder may contribute to the disruption of urinary bladder function in the aged. That is, this research clearly demonstrates that age-related alterations in the autonomic responsiveness of the rat urinary bladder exist. However, many questions remain unanswered. For example, whether the observed changes in isotonic contractions of the bladder affect the storage and expulsion functions of the bladder is not known and is probably the most important unresolved issue. First, and foremost, future experiments should examine the aged rat bladder cystometrically to determine whether functional changes similar to humans exist. These experiments would simply involve catheterizing an anesthetized, or possibly a restrained unanesthetized rat, and performing a cystometric evaluation of bladder function, including the determination of residual urine, speed of urine flow during voiding, bladder capacity, and possible presence of detrusor contractions during filling (uninhibited bladder
contractions) in young and old rats. Next, the effects of various agonists and antagonists infused intravenously on these parameters could be measured to determine whether the observed changes in muscarinic and alpha adrenergic receptor responsiveness with age cause the changes in function predicted in section 5.3. These studies would also allow the examination of age on other factors which affect bladder function, e.g. sensory reflex pathways and CNS control. Also, studies on the effects of age on the bladder pressure changes elicited by these agonists could be simplified by cystometrically examining the isolated intact bladder in vitro.

The most intriguing observation of the present studies, and probably the most difficult to explain, is the paradoxical decrease in the density of muscarinic receptors in the bladder neck, where an increase in muscarinic responsiveness was observed. The possibility that the radiolabelled antagonist, \(^{3}\text{H}\)QNB, binds to a different site than muscarinic agonists and, therefore, does not approximate the number of agonist binding sites was examined in a preliminary experiment (data not included). Bethanechol displaced all of the radiolabelled antagonist in homogenates of both 7 and 27 mo bladder necks, indicating that \(^{3}\text{H}\)QNB and bethanechol bind to the same site. Hence, the fewer number of receptors in the 27 mo bladder necks appear to be capable of a greater biochemical response than those of the 7 mo bladder necks.

Both KCl and muscarinic agonists produce contractions of smooth muscles by raising intracellular calcium. Since no change was observed with age in the maximum contractions elicited by KCl, it appears that the smooth muscle contractile machinery (actin, myosin, etc.) is not
altered by age. Hence, alterations most likely occur somewhere in the cascade of biochemical events which follow muscarinic receptor occupation and which precede the activation of myosin light chain kinase by calcium. Therefore, future experiments should examine muscarinic receptor-mediated biochemical responses (e.g. formation of phosphatic acid or myoinositol phosphate, calcium flux) in young and old rats in an attempt to elucidate the mechanism by which an increase in muscarinic agonist-induced contractions occurs.

Elucidation of a mechanism by which an increase in the alpha adrenoceptor responsiveness occurs in the bladder body also represents a formidable experimental problem. Although utilization of receptor binding techniques was unsuccessful in the present studies, other radiolabelled ligands are available and might prove to be useful. For example, \(^{3}\text{H}\)-prazosin has a specific activity 4 times higher than the \(^{3}\text{H}\)-DHE used in the present attempt to label receptors. Moreover, the technical problems associated with \(^{125}\text{I}\)-HEAT binding (see Section 3.3) could be avoided with the use of \(^{3}\text{H}\)-prazosin. If receptor density can be determined, then \(^{3}\text{H}\)-prazosin is a good choice of ligands not yet tested. If alpha adrenoceptor density can be determined with this or any other ligand, the data should be cautiously interpreted since changes in receptor densities do not correlate with changes in responsiveness of the muscarinic receptor system. Examination of the biochemical events following alpha adrenoceptor activation in tissues from young and old bladders, similar to those experiments described for the muscarinic receptor system, may prove to be most valuable in terms of understanding the cause of the enhanced receptor-mediated contractility of the aged bladder body.
Finally, many other aspects of the control of the urinary bladder should be examined. For example, age-induced changes in the activity of the autonomic nerves innervating the bladder may cause the postsynaptic alterations observed in the present studies. Furthermore, changes in the afferent input coming from proprioceptors in the bladder smooth muscle or from descending central neurons could be responsible for any alterations in autonomic nerve activity. It is rather obvious that the present research has only "scratched the surface" in terms of understanding the effects of age on the function of the urinary bladder.
6.0 Major Findings of this Research

The following are conclusions supported by the results of this research.

1. Regionally specific changes with age occur in both the muscarinic and alpha adrenergic receptor systems of the F344 rat urinary bladder.

2. No age-dependent changes in the maximum contractions induced by potassium chloride were observed in the bladder body or the bladder neck. Therefore, the capacity of these tissues to isotonically contract does not change with age.

3. An age-related increase in the maximum contractions elicited by muscarinic agonists was observed in the bladder neck while no such change was observed in the bladder body. In the bladder neck, enhanced contractions occurred not only at the Emax, but all along the cumulative concentration-response.
4. A paradoxical decrease in the density of muscarinic receptors occurred in the bladder neck between the ages of 16 and 27 mo, when the largest increase in the muscarinic contractile responsiveness was observed. In the bladder body, an increase in the density of muscarinic receptors was observed.

5. An age-related increase in the alpha adrenoceptor responsiveness of the bladder body was observed, while no such change was observed in the bladder neck. In the bladder body, enhanced contractions occurred not only at the Emax, but all along the cumulative concentration-response.

6. Contractions of the bladder body elicited by alpha adrenoceptor agonists are mediated by alpha_1 adrenoceptors. Furthermore, the enhanced contractions of the aged bladder body elicited by alpha adrenoceptor agonists are also mediated by alpha_1 adrenoceptors. Contractions of the bladder neck are mediated by alpha_1 adrenoceptors; there is a possible existence of a small population of alpha_2 receptors on smooth muscle of the bladder neck.

7. No age-related changes in the ED50 values of muscarinic or alpha adrenergic agonists were observed in the bladder body or the bladder neck. Furthermore, no change in the Kd of [H]QNB was observed in either bladder region.
6.1 General Implications

This research dealt specifically with the effects of age on the autonomic responsiveness of the rat urinary bladder. However, in a more broad sense, these studies were an examination of the autonomic neuropharmacology of the aging process. In this regard, the results of this research effort raise questions concerning the validity of certain generalizations of the aging process. Probably the most widely held contention concerning the neuropharmacology of aging is that a general decline in the sensitivity of organ systems occurs with age (Frolikis et al., 1973; Collins et al., 1980; Brodde et al., 1982; Simpkins et al., 1983). In fact, following a perusal of the abundance of literature on the effects of age on neurotransmitter responsiveness of a variety of tissues (see Roth and Hess, 1982 for review), one may gather that indeed this is the case. In fact, cell systems which undergo declines in receptor number and biochemical responsiveness, e.g. a decline in adrenergic receptor density in platelets (Brodde et al., 1982), are purported to be model systems of the effects of age on the reduced adrenergic responsiveness of the elderly. The fact that the bladder actually undergoes increases in responsiveness to autonomic agents, indicates that generalizations of the effects of age on the autonomic responsiveness of organs should be reconsidered. Furthermore, age-related changes are regionally specific in the bladder itself, demonstrating the need for more detailed studies on different areas of organs, especially in areas that differ in terms of innervation, in terms of distribution of autonomic receptors, or differ in terms of anatomy.
Another common age study is demonstration of changes in receptor density unaccompanied by functional studies (Shocken and Roth, 1977; Landmann et al., 1981; Brodde et al., 1982). Obviously the results of the present study demonstrate that it is definitely insufficient to examine only receptor density, since such changes may be opposite to changes in the functional responsiveness of the tissue. Furthermore, the demonstration of a receptor density change is often considered the causative factor for a parallel change in the functional responsiveness of a tissue (Lai et al., 1983). However, a change in receptor density with age in the bladder may only reflect a change in the spare receptor population, and therefore, may have no functional consequence. Thus, more detailed studies are needed in aging research, such as that by Ito et al. (1982) which examine not just functional response and receptor number, but also biochemical events which occur between receptor activation and functional response. Such studies on the bladder are currently underway.

Finally, this research demonstrates that it would be erroneous to assume that a given trend in an apparent age-related effect will continue linearly throughout the lifespan of the animal. For example, a small increase in responsiveness of the bladder neck to bethanechol and oxotremorine between 7 and 16 mo was followed by a much greater increase between 16 and 27 mo (see Figure 12). A more striking example is the reversal of the direction of change with age in bladder neck muscarinic receptor density, from an increase between ages 7 and 16 mo to a decrease between ages 16 and 27 mo (see Figure 26). Hence, aging studies should utilize as many age groups as economically
possible, with a very minimum of 3 age groups, in order to avoid inaccurate generalizations of trends in age-related changes.

The neuropharmacology of aging is a rapidly expanding field of research which has so far generated many more observations than explanations. Understanding the underlying physiological mechanisms resulting in observed age-associated alterations may some day lead to interventions of the aging process and the ultimate improvement of the quality of elderly life.


APPENDIX A

COMLOT SAS

**************************************************************;
*;
* THE COMLOT PROGRAM CONVERTS MILLIMETERS PEN DISPLACEMENT MEASURED*;
* BY THE COMLOT DIGITIZER TO MILLIMETERS TISSUE MOVEMENT. *
* COMLOT AVERAGES RESPONSES FROM TWO SUCCESSIVE DOSE RESPONSE *
* CURVES WHICH HAVE BEEN READ BY THE COMLOT DIGITIZER. *
* THE DATA GENERATED BY COMLOT IS IN A FORMAT EASILY ADJUSTED *
* TO FIT THAT WHICH IS NEEDED FOR USE IN BOTH ITON1 AND ITON2. *
* DATA FILES GENERATED BY THE DIGITIZER ARE IN THE FORM: *
* FILENAME FILETYPE *
* 12345678 DIGDATA *
* WHERE 1=TISSUE TYPE, 2=AGE GROUP, 3=TISSUE NO., 4567=DRUG *
* AND 8=C DENOTING A COMLOT DIGDATA FILE. *
* DATA FILES GENERATED BY THE DIGITIZER ARE FILETYPED DIGDATA *
* AUTOMATICALLY, THESE FILES WILL BE READ DIRECTLY BY COMLOT *
* BY SUBSTITUTING THE FILENAME OF THE DIGDATA FILE IN THE *
* 4 LINES BELOW WHERE filename APPEARS. FURTHERMORE THE LINE *
* AFTER THE CARDS STATEMENT BELOW WILL BE CHANGED TO IDENTIFY THE *
* DIGDATA SET ABOUT TO BE COMPUTED. OUTPUT OF THIS FILE WILL BE *
* IN THE FORM NECESSARY FOR USE BY THE ITON1, ITON2, AND STAT *
* PROGRAMS I.E. COMLOT PRODUCES A PERMANENT FILE CONTAINING *
* THE COMPUTED MM TISSUE MOVEMENTS FOR EACH CONCENTRATION AND *
* NAMES THIS DATA SET filename DATA.
* ADDITIONALLY THE COMLOT PROGRAM ADJUSTS FOR SENSITIVITY CHANGES*;
* MADE DURING THE EXPERIMENT. THE SENSITIVITY CHANGE MADE FOR *
* A GIVEN CONCENTRATION MUST BE SPECIFIED IN COLUMNS 2-3 OF *
* OF THE DIGDATA FILE, IE. *
* COLUMNS
* 1 2-3 4-10 11-ETC. DATUM CONCENTRATION FACTOR X COORDINATES RESPONSE *
* THE FACTOR IS A 2 DIGIT NUMBER WITH ONE DECIMAL POINT (NOT *
* INCLUDED BUT READ BY THE PROGRAM). USE OF COMLOT REQUIRES THE *
* USER TO REPLACE THE COLUMN 2 OF DIGDATA FILE (+ SIGN) *
* WITH A 1 IF NO SENSITIVITY CHANGE WAS USED, 2 IF SENSITIVITY *
* WAS HALVED, 2.5 IF SENSITIVITY WAS CHANGED FROM 200 TO 500, *
* ETC. (THE DECIMALS MUST BE OMITTED! I.E. 2.5 = 25. *
*
* DIGDATA FILES MUST BE CREATED IN THE FOLLOWING ORDER FOR COMLOT*
* TO READ THEM CORRECTLY.**
* LINE NUMBER      DATUM
*  1                L2/L1*calibration
*  2                calibration height
*  3                calibration baseline
*  4                baseline
*  5-14             successive concentration-responses
*  15               L2/L1*calibration(save as 1)
*  16               calibration height
*  17               calibration baseline
*  18               baseline
*  19-28            successive concentration-responses
*                    of second curve
*  29               L2/L1*calibration
*  30               calibration height
*  31               calibration baseline
*  32               baseline
*  33               response to 0.1 M KCl
*  34               L2/L1*calibration
*  35               calibration height
*  36               calibration baseline
*  37               baseline
*  38               response to 0.3 M KCl
*
* by GREGORY A. ORDWAY AND LANE J. WALLACE 10/13/83
*
*****************************************************************************

OPTIONS LINESIZE=80;
TITLE SY4PDES;
TITLE2 COMPLOT;
DATA SCRIPT;
INPUT DATE DATE7. CHANNEL TISSUE $ ORDER R_MAX 4.2 DRUG $25.;
DUMMY = 2;
CARDS;
QBAUG84  1 SPHINC 4 100 YS-PILO-DESENS
CMS FILEDEF BOZO DISK filename DIGDATA A1;
CMS FILEDEF LOGO DISK filename DATA A1 (DISP MOD);
CMS FILEDEF 20 DISK filename NOTEBOOK A1 (DISP MOD);
CMS FILEDEF 21 DISK filename NOTEBOOKK A1 (DISP MOD);
DATA ONE;
INFILE BOZO FIRSTOBS=1 OBS=4;
INPUT CONST 1-8 / @11 CALH 7.3 / @11 CALB 7.3 / @11 BASE 7.3;
DUMMY = 1;
DATA TWO;
INFILE BOZO FIRSTOBS=5 OBS=14;
INPUT CONC 1 @2 FACT 2.1 @11 RESP 7.3;
DUMMY = 1;
IF CONC = 4 THEN CONC = 3E-9;
IF CONC = 5 THEN CONC = 1E-8;
IF CONC = 6 THEN CONC = 3E-8;
IF CONC = 7 THEN CONC = 1E-7;
IF CONC = 8 THEN CONC = 3E-7;
IF CONC = 9 THEN CONC = 1E-6;
IF CONC = 0 THEN CONC = 3E-6;
IF CONC = 1 THEN CONC = 1E-5;
IF CONC = 2 THEN CONC = 3E-5;
IF CONC = 3 THEN CONC = 1E-4;
DATA THREE; MERGE ONE TWO; BY DUMMY;
LTM=CONST/(CALH-CALB)*(RESP-BASE)*FACT;
PROC SORT; BY CONC;
DATA ONEA;
INFILE BOZO FIRSTOBS=15 OBS=18;
INPUT CONST1 1-8 / @11 CALH1 7.3 / @11 CALB1 7.3 / @11 BASE1 7.3;
DUMMY = 2;
DATA TWOA;
INFILE BOZO FIRSTOBS=19 OBS=28;
INPUT CONC 1 @2 FACT1 2.1 @11 RESP1 7.3;
DUMMY = 2;
IF CONC = 4 THEN CONC = 3E-9;
IF CONC = 5 THEN CONC = 1E-8;
IF CONC = 6 THEN CONC = 3E-8;
IF CONC = 7 THEN CONC = 1E-7;
IF CONC = 8 THEN CONC = 3E-7;
IF CONC = 9 THEN CONC = 1E-6;
IF CONC = 0 THEN CONC = 3E-6;
IF CONC = 1 THEN CONC = 1E-5;
IF CONC = 2 THEN CONC = 3E-5;
IF CONC = 3 THEN CONC = 1E-4;
DATA THREEA; MERGE ONEA TWOA; BY DUMMY;
LTM1=CONST1/(CALH1-CALB1)*(RESP1-BASE1)*FACT1;
PROC SORT; BY CONC;
DATA TOGETHER; MERGE THREE THREEA; BY CONC;
   MLTM = SUM (LTM, LTM1)/N(LTM, LTM1);
FORMAT CONC E8. LTM1 5.2;
PROC PRINTTO UNIT=20;
PROC PRINT;
VAR CONST CALH CALB BASE RESP CALH1 CALB1 BASE1 RESP1
   CONC LTM LTM1 MLTM;
PROC PRINTTO;
DATA FOURA;
   INPUT CONC MLTM; DUMMY = 2; CARDS;
1E-9 .
3E-9 .
1E-8 .
3E-8 .
1E-7 .
3E-7 .
1E-6 .
3E-6 .
1E-5 .
3E-5 .
1E-4 .
3E-4 .
1E-3 .
PROC SORT; BY CONC;
DATA SEVEN; MERGE FOURA TOGETHER; BY CONC;
PROC SORT; BY DUMMY;
DATA EIGHT;
INFILE BOZO FIRSTOBS=29 OBS=32;
INPUT CONSTK4 1-8 / @11 CALHK4 7.3 / @11 CALBK4 7.3 / @11 BASEK4 7.3;
DUMMY = 1;
DATA NINE;
INFILE BOZO FIRSTOBS=33 OBS=33;
INPUT CONC 1 @2 FACT2 2.1 @11 RESPK4 7.3;
DUMMY = 1;
DATA TEN; MERGE EIGHT NINE; BY DUMMY;
DUMMY = 2;
  R_MAX4=CONSTK4/(CALHK4-CALBK4)*(RESPK4-BASEK4)*FACT2;
PROC SORT; BY CONC;
FORMAT R_MAX4 5.2;
DATA ELEVEN;
INFILE BOZO FIRSTOBS=34 OBS=37;
INPUT CONSTK5 1-8 / @11 CALHK5 7.3 /@11 CALBK5 7.3 / @11 BASEK5 7.3;
DUMMY = 1;
DATA TWELVE;
INFILE BOZO FIRSTOBS=38 OBS=38;
INPUT CONC 1 @2 FACT3 2.1 @11 RESPK5 7.3;
DUMMY = 1;
DATA THIRTEEN; MERGE ELEVEN TWELVE; BY DUMMY;
DUMMY = 2;
  R_MAX5=CONSTK5/(CALHK5-CALBK5)*(RESPK5-BASEK5)*FACT3;
FORMAT R_MAX5 5.2;
PROC SORT; BY CONC;
DATA FOURTEEN; MERGE TEN THIRTEEN; BY DUMMY;
R_MAX=MAX(R_MAX4,R_MAX5);
PROC PRINTTO UNIT=21;
PROC PRINT;
VAR CALHK4 CALBK4 R_MAX4 BASEK4 RESPK4
  CALHK5 CALBK5 BASEK5 RESPK5 R_MAX5;
PROC PRINTTO;
DATA FOUR; MERGE SEVEN SCRIPT FOURTEEN(KEEP=DUMMY R_MAX); BY DUMMY;
DATA NULL; SET FOUR;
FILE LOCO;
IF CONC = 1E-9 THEN PUT @1 DATE DATE7.
  @9 CHANNEL 1.
  @11 TISSUE $6.
  @18 ORDER 1.
  @20 R_MAX 4.2
  @26 DRUG $25./
  @1 MLTM 5.2 @;
IF CONC = 3E-9 THEN PUT @7 MLTM 5.2 @;
IF CONC = 1E-8 THEN PUT @13 MLTM 5.2 @;
IF CONC = 3E-8 THEN PUT @18 MLTM 5.2 @;
IF CONC = 1E-7 THEN PUT @24 MLTM 5.2 @;
IF CONC = 3E-7 THEN PUT @30 MLTM 5.2 @;
IF CONC = 1E-6 THEN PUT @36 MLTM 5.2 @;
IF CONC = 3E-6 THEN PUT @42 MLTM 5.2 @;
IF CONC = 1E-5 THEN PUT @48 MLTM 5.2 @;
IF CONC = 3E-5 THEN PUT @54 MLTM 5.2 @;
IF CONC = 1E-4 THEN PUT @60 MLTM 5.2 @;
IF CONC = 3E-4 THEN PUT @66 MLTM 5.2 @;
IF CONC = 1E-3 THEN PUT @72 MLTM 5.2 @;
*******************END OF COMPLOT PROGRAM***************************
APPENDIX B

ITON1 SAS

*********************************************************
* CURVE FITTING PROCEDURE TO THE LOGISTIC FUNCTION *
* MODULE INPUTS DATA CONTAINING DAILY RESULTS. *
* ALSO DRAWS CURVES OF BOTH THE RAW DATA FOR EACH POINT *
* AND THE PREDICTED VALUE OF THAT POINT AS CALCULATED BY *
* THE LOGISTIC FUNCTION. *
* DATA INPUT MUST BE IN THE FORM IDENTIFIED AS FOLLOWS. *
* THIS FORM IS THE OUTPUT FROM COMPLOT, THE DATA FILE *
* BEING filename DATA.
* The 1st line identifies data of next line in the *
* following order:
* date/tissue number/tissue/bath number/maximum contraction to *
* KCL or drug/drug or age and drug identification *
* EXAMPLE:
* 07AUG84 1 NECK 1 2.74 YN-PHENYLEPHRINE *
* *
* The 2nd line contains the mm tissue movement *
* *
* AN EXAMPLE OF A DATA FILE FOLLOWS THE CARDS STATEMENT BELOW. *
* AS WRITTEN THE PROGRAM Sorts DATA ACCORDING TO THE DATE *
* AND DRUG: THIS CAN EASILY BE MODIFIED. *
* *
* ITON1 written by PETER J. RICE, modified here by G.A. ORDWAY *
*********************************************************
OPTIONS LABEL NODATE NONUMBER NOSOURCE ERRORABEND;
CMS FILEDEF 20 DISK NONLIN2O LISTING A1;

***********************************************************
DATA ALL1;
INPUT @1 DATE DATE7.
@9 CHANNL 1.
@11 TISSUE $6.
@18 ORDER 1.
  R_MAX 20-24
@26 DRUG $25.

  / EFFECT3-EFFECT15;
  CONC1 = 1E-10 ; EFFECT1 = . ;
  CONC2 = 3E-10 ; EFFECT2 = . ;
  CONC3 = 1E-09 ;
  CONC4 = 3E-09 ;

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CARDS;
06JUL84 1 NECK 1 5.28 YN-OXOTREMORINE
 0.00 0.03 0.09 0.15 0.33 0.81 1.35 1.53 1.65 . . . .
06JUL84 1 NECK 1 2.61 MN-OXOTREMORINE
 0.00 0.00 0.00 0.06 0.26 0.40 0.60 0.72 0.72 . . . .
06JUL84 1 NECK 1 6.03 ON-OXOTREMORINE
 0.00 0.03 0.12 0.42 1.08 1.80 2.28 2.40 2.46 . . . .
;
******************************************************************************;
** DATA SET configuration of ALL1 **;
** **;
** DATE CHANNEL TISSUE _MAX DRUG EFFECT1- **;
** EFFECT15 _CONC1-15 **;
** **
******************************************************************************;
******************************************************************************;
*** Module to fit Concentration - Effect data ***;
*** TO THE LOGISTIC FUNCTION ***;
******************************************************************************;
DATA ALL2;
SET ALL1;
DROP EFFECT1-EFFECT15 CONC1-Conc15;
EFFECT=EFFECT1; CONC=CONC1; OUTPUT;
EFFECT=EFFECT2; CONC=CONC2; OUTPUT;
EFFECT=EFFECT3; CONC=CONC3; OUTPUT;
EFFECT=EFFECT4; CONC=CONC4; OUTPUT;
EFFECT=EFFECT5; CONC=CONC5; OUTPUT;
EFFECT=EFFECT6; CONC=CONC6; OUTPUT;
EFFECT=EFFECT7; CONC=CONC7; OUTPUT;
EFFECT=EFFECT8; CONC=CONC8; OUTPUT;
EFFECT=EFFECT9; CONC=CONC9; OUTPUT;
EFFECT=EFFECT10; CONC=CONC10; OUTPUT;
EFFECT=EFFECT11; CONC=CONC11; OUTPUT;
EFFECT=EFFECT12; CONC=CONC12; OUTPUT;
EFFECT=EFFECT13; CONC=CONC13; OUTPUT;
EFFECT=EFFECT14; CONC=CONC14; OUTPUT;
EFFECT=EFFECT15; CONC=CONC15; OUTPUT;
FORMAT EFFECT 6.4 CONC E8.1;
PROC SORT DATA=ALL2; BY DATE CHANNEL ORDER;
DATA TEMP;
SET ALL2; IF EFFECT AND EFFECT. ;
PROC SORT DATA=TEMP; BY DATE CHANRL ORDER ;
PROC PRINTTO UNIT=20 NEW. ;
PROC NLMIXED DATA=TEMP BEST=1 CONVERGENCE=1E-12
ITER=46 METHOD=MARQUEDT;
BY DATE CHANRL ORDER ;
TITLE4 NONLINEAR CURVE FITTING ;
TITLE5 CONCENTRATION _ EFFECT CURVE TO THE LOGISTIC FUNCTION ;
PARAMETERS K = 1E-09 , 1E-07 , 1E-05
M = 10 , 20 , 30
P = 1.0 ;
BOUNDS O < K < 1
O < M < 100
O < P < 5 ;
MODEL EFFECT = M * CONC**P / (CONC**P + K**P) ;
DER.K = -M * CONC**P * (CONC**P + K**P)**(-2) * P * K**(P-1) ;
DER.M = CONC**P / (CONC**P + K**P) ;
DER.P = M * ((CONC**P + K**P)**(-1) * (CONC**P * LOG(CONC))
- (CONC**P * (CONC**P + K**P)**(-2) * (CONC**P *
LOG(CONC) + K**P * LOG(K)))) ;
FORMAT DATE DATE7• 5
OUTPUT OUT = ALL2
PARMS = K M P
PREDICTED = EA_PRE
ESS = RSS ;
PROC DELETE DATA=TEMP ;
PROC PRINTTO ;
PROC PLOT DATA = ALL2; BY DATE CHANRL ORDER ;
PLOT EFFECT*CONC='+' EA_PRE*CONC='X'/OVERLAY HAXIS=1E-9 1E-8 1E-7 1E-6 1E-5 1E-4 1E-3;
PROC SORT DATA=ALL1; BY DATE CHANRL ORDER ;
PROC SORT DATA=ALL2; BY DATE CHANRL ORDER ;
DATA ALL1 ;
UPDATE ALL1 ALL2 ; BY DATE CHANRL ORDER ;
DROP EFFECT CONC EA_PRE ;
*** **************************** **************************** **************************** **************************** ;
DATA _NULL_ ;
SET ALL1 ;
FILE PRINT N=PS NOTITLES ; BY DATE CHANRL ORDER ;
L=(7 + 7*CHANRL) ; LO=(7 + 7*CHANRL + ORDER); IF FIRST.DATE THEN DO ;
PUT PAGE ;
PUT #7 @71 DATE DATES9 .
#9 @25 'PARAMETERS OF THE LOGISTIC FUNCTION'
#10 @24 'EFFECT = MAX * CONC**P / (CONC**P + K**P)' ;
END ;
IF FIRST.CHANRL THEN DO ;
PUT #L @11 'CHANNEL #'
@21 CHANRL
DATA _NULL_;  
SET ALL;  BY DATE CHANNL ORDER ;  
FILE PRINT N=PS NOTITLES ;  
L= 6*CHANNL + 6 ;  
IF FIRST.DATE THEN DO ;  
PUT PAGE ;  
PUT #1 @1 110* ' ;  
#7 @42 'STATISTICAL ANALYSIS SYSTEM'  
@94 DATE DATE9.  
#9 @40 'EFFECT -- MOVEMENT IN MILLIMETERS' ;  
END ;  
IF FIRST.CHANNL THEN DO ;  
PUT #L @10 '#'  
@11 CHANNL  
@14 '1E-10' @20 '3E-10'  
@26 '1E-09' @32 '3E-09'  
@38 '1E-08' @44 '3E-08'  
@50 '1E-07' @56 '3E-07'  
@62 '1E-06' @68 '3E-06'  
@74 '1E-05' @80 '3E-05'  
@86 '1E-04' @92 '3E-04'  
@98 '1E-03' ;  
END ;  
PUT ORDER 10  
EFFECT1 14-18 2  EFFECT2 20-24 2  
EFFECT3 26-30 2  EFFECT4 32-36 2  
EFFECT5 38-42 2  EFFECT6 44-48 2  
EFFECT7 50-54 2  EFFECT8 56-60 2  
EFFECT9 62-66 2  EFFECT10 68-72 2  
EFFECT11 74-78 2  EFFECT12 80-84 2  
EFFECT13 85-90 2  EFFECT14 92-96 2  
EFFECT15 98-102 2  
;
IF LAST.DATE THEN DO ;
   PUT #52 @1 110*'_'
;  
END ;
*****************************************************************************;
APPENDIX C

PREDICTED VALUES

Below is output from ITON1 SAS (APPENDIX B) for a single concentration response. Both the observed effect in mm tissues movement at a given drug concentration (X) and the predicted value calculated by ITON1 (+) are plotted.
APPENDIX D

ITON2 SAS

**********************************************************************;
* PROGRAM "ITON2" COMPUTES drug-induced response *;
* AS PERCENTS OF THE MAXIMUM CONTRACTIONS ELICITED BY *;
* THE NONSPECIFIC CONTRACTILE AGENT KCL. *;
* *;
* INPUT FOR ITON2 IS IDENTICAL TO THE DATA *;
* FILES USED IN ITON1. DATA FILES SHOULD BE INSERTED JUST *;
* AFTER THE CARDS STATEMENT. *
* *
************* PROGRAM WRITTEN BY PETER J. RICE ***************;
OPTIONS LABEL ERRORABEND NODATE NONUMBER NOSOURCE OVP ;
**********************************************************************;
DATA ALL1 ;
INPUT @1 DATE DATE7. 
@9 CHANNL 1. 
@11 TISSUE $6. 
@18 ORDER 1. 
R MAX 20-24 
@26 DRUG $25. 
/
EFFECT3-EFFECT15 ;

EFFECT1 = . ;
EFFECT2 = . ;

PERCNT1 = EFFECT1 / R MAX * 100 ; CONC1 = 1E-10 ;
PERCNT2 = EFFECT2 / R MAX * 100 ; CONC2 = 3E-10 ;
PERCNT3 = EFFECT3 / R MAX * 100 ; CONC3 = 1E-09 ;
PERCNT4 = EFFECT4 / R MAX * 100 ; CONC4 = 3E-09 ;
PERCNT5 = EFFECT5 / R MAX * 100 ; CONC5 = 1E-08 ;
PERCNT6 = EFFECT6 / R MAX * 100 ; CONC6 = 3E-08 ;
PERCNT7 = EFFECT7 / R MAX * 100 ; CONC7 = 1E-07 ;
PERCNT8 = EFFECT8 / R MAX * 100 ; CONC8 = 3E-07 ;
PERCNT9 = EFFECT9 / R MAX * 100 ; CONC9 = 1E-06 ;
PERCNT10 = EFFECT10 / R MAX * 100 ; CONC10 = 3E-06 ;
PERCNT11 = EFFECT11 / R MAX * 100 ; CONC11 = 1E-05 ;
PERCNT12 = EFFECT12 / R MAX * 100 ; CONC12 = 3E-05 ;
PERCNT13 = EFFECT13 / R MAX * 100 ; CONC13 = 1E-04 ;
PERCNT14 = EFFECT14 / R MAX * 100 ; CONC14 = 3E-04 ;
PERCNT15 = EFFECT15 / R MAX * 100 ; CONC15 = 1E-03 ;
FORMAT PERCNT1 3.0 PERCNT2 3.0 PERCNT3 3.0 PERCNT4 3.0
DATA ALL1;
SET ALL1;
*** LOGARITHMIC INTERPOLATION TO COMPUTE ED VALUES ***;
* OPTIONS NOSOURCE;*

IF PERCNT1 <= 10 AND PERCNT2 >= 10 THEN LGED10 =
   \(\log_{10}(CONC1) + ((10 - \text{PERCNT1}) / (\text{PERCNT2} - \text{PERCNT1}))\) \* (\(\log_{10}(CONC2) - \log_{10}(CONC1)\)) ;
IF PERCNT2 <= 10 AND PERCNT3 >= 10 THEN LGED10 =
   \(\log_{10}(CONC2) + ((10 - \text{PERCNT2}) / (\text{PERCNT3} - \text{PERCNT2}))\) \* (\(\log_{10}(CONC3) - \log_{10}(CONC2)\)) ;
IF PERCNT3 <= 10 AND PERCNT4 >= 10 THEN LGED10 =
   \(\log_{10}(CONC3) + ((10 - \text{PERCNT3}) / (\text{PERCNT4} - \text{PERCNT3}))\) \* (\(\log_{10}(CONC4) - \log_{10}(CONC3)\)) ;
IF PERCNT4 <= 10 AND PERCNT5 >= 10 THEN LGED10 =
   \(\log_{10}(CONC4) + ((10 - \text{PERCNT4}) / (\text{PERCNT5} - \text{PERCNT4}))\) \* (\(\log_{10}(CONC5) - \log_{10}(CONC4)\)) ;
IF PERCNT5 <= 10 AND PERCNT6 >= 10 THEN LGED10 =
   \(\log_{10}(CONC5) + ((10 - \text{PERCNT5}) / (\text{PERCNT6} - \text{PERCNT5}))\) \* (\(\log_{10}(CONC6) - \log_{10}(CONC5)\)) ;
IF PERCNT6 <= 10 AND PERCNT7 >= 10 THEN LGED10 =
   \(\log_{10}(CONC6) + ((10 - \text{PERCNT6}) / (\text{PERCNT7} - \text{PERCNT6}))\) \* (\(\log_{10}(CONC7) - \log_{10}(CONC6)\)) ;
IF PERCNT7 <= 10 AND PERCNT8 >= 10 THEN LGED10 =
   \(\log_{10}(CONC7) + ((10 - \text{PERCNT7}) / (\text{PERCNT8} - \text{PERCNT7}))\) \* (\(\log_{10}(CONC8) - \log_{10}(CONC7)\)) ;
IF PERCNT8 <= 10 AND PERCNT9 >= 10 THEN LGED10 =
   \(\log_{10}(CONC8) + ((10 - \text{PERCNT8}) / (\text{PERCNT9} - \text{PERCNT8}))\) \* (\(\log_{10}(CONC9) - \log_{10}(CONC8)\)) ;
IF PERCNT9 <= 10 AND PERCNT10 >= 10 THEN LGED10 =
   \(\log_{10}(CONC9) + ((10 - \text{PERCNT9}) / (\text{PERCNT10} - \text{PERCNT9}))\) \* (\(\log_{10}(CONC10) - \log_{10}(CONC9)\)) ;
IF PERCNT10 <= 10 AND PERCNT11 >= 10 THEN LGED10 =
   \(\log_{10}(CONC10) + ((10 - \text{PERCNT10}) / (\text{PERCNT11} - \text{PERCNT10}))\) \* (\(\log_{10}(CONC11) - \log_{10}(CONC10)\)) ;
IF PERCNT11 <= 10 AND PERCNT12 >= 10 THEN LGED10 =
   \(\log_{10}(CONC11) + ((10 - \text{PERCNT11}) / (\text{PERCNT12} - \text{PERCNT11}))\) \* (\(\log_{10}(CONC12) - \log_{10}(CONC11)\)) ;
IF PERCNT12 <= 10 AND PERCNT13 >= 10 THEN LGED10 =
   \(\log_{10}(CONC12) + ((10 - \text{PERCNT12}) / (\text{PERCNT13} - \text{PERCNT12}))\) \* (\(\log_{10}(CONC13) - \log_{10}(CONC12)\)) ;
*(LOG10(CONC13)-LOG10(CONC12)) ;
IF PERCNT13<10 AND PERCNT14>=10 THEN LGED10 =
LOG10(CONC13)+((10-PERCNT13)/(PERCNT14-PERCNT13))
*(LOG10(CONC14)-LOG10(CONC13)) ;
IF PERCNT14<10 AND PERCNT15>=10 THEN LGED10 =
LOG10(CONC14)+((10-PERCNT14)/(PERCNT15-PERCNT14))
*(LOG10(CONC15)-LOG10(CONC14)) ;
ED10 = 10**LGED10 ;
NLED10 = - LGED10 ;
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IF PERCNT1<20 AND PERCNT2 >=20 THEN LGED20 =
LOG10(CONC1 )+((20-PERCNT1 )/(PERCNT2 -PERCNT1 ))
*(LOG10(CONC2 )-LOG10(CONC1 )) ;
IF PERCNT2<20 AND PERCNT3 >=20 THEN LGED20 =
LOG10(CONC2 )+((20-PERCNT2)/(PERCNT3-PERCNT2 ))
*(LOG10(CONC3 )-LOG10(CONC2 )) ;
IF PERCNT3<20 AND PERCNT4 >=20 THEN LGED20 =
LOG10(CONC3 )+((20-PERCNT3)/(PERCNT4-PERCNT3 ))
*(LOG10(CONC4 )-LOG10(CONC3 )) ;
IF PERCNT4<20 AND PERCNT5 >=20 THEN LGED20 =
LOG10(CONC4 )+((20-PERCNT4)/(PERCNT5-PERCNT4 ))
*(LOG10(CONC5 )-LOG10(CONC4 )) ;
IF PERCNT5<20 AND PERCNT6 >=20 THEN LGED20 =
LOG10(CONC5 )+((20-PERCNT5)/(PERCNT6-PERCNT5 ))
*(LOG10(CONC6 )-LOG10(CONC5 )) ;
IF PERCNT6<20 AND PERCNT7 >=20 THEN LGED20 =
LOG10(CONC6 )+((20-PERCNT6)/(PERCNT7-PERCNT6 ))
*(LOG10(CONC7 )-LOG10(CONC6 )) ;
IF PERCNT7<20 AND PERCNT8 >=20 THEN LGED20 =
LOG10(CONC7 )+((20-PERCNT7)/(PERCNT8-PERCNT7 ))
*(LOG10(CONC8 )-LOG10(CONC7 )) ;
IF PERCNT8<20 AND PERCNT9 >=20 THEN LGED20 =
LOG10(CONC8 )+((20-PERCNT8)/(PERCNT9-PERCNT8 ))
*(LOG10(CONC9 )-LOG10(CONC8 )) ;
IF PERCNT9<20 AND PERCNT10>=20 THEN LGED20 =
LOG10(CONC9 )+((20-PERCNT9)/(PERCNT10-PERCNT9 ))
*(LOG10(CONC10)-LOG10(CONC9 )) ;
IF PERCNT10<20 AND PERCNT11>=20 THEN LGED20 =
LOG10(CONC10)+((20-PERCNT10)/(PERCNT11-PERCNT10))
*(LOG10(CONC11)-LOG10(CONC10)) ;
IF PERCNT11<20 AND PERCNT12>=20 THEN LGED20 =
LOG10(CONC11)+((20-PERCNT11)/(PERCNT12-PERCNT11))
*(LOG10(CONC12)-LOG10(CONC11)) ;
IF PERCNT12<20 AND PERCNT13>=20 THEN LGED20 =
LOG10(CONC12)+((20-PERCNT12)/(PERCNT13-PERCNT12))
*(LOG10(CONC13)-LOG10(CONC12)) ;
IF PERCNT13<20 AND PERCNT14>=20 THEN LGED20 =
LOG10(CONC13)+((20-PERCNT13)/(PERCNT14-PERCNT13))
*(LOG10(CONC14)-LOG10(CONC13)) ;
IF PERCNT14<20 AND PERCNT15>=20 THEN LGED20 =
LOG10(CONC14)+((20-PERCNT14)/(PERCNT15-PERCNT14))
*(LOG10(CONC15)-LOG10(CONC14)) ;
ED20 = 10**LGED20
NLED20 = - LGED20

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IF PERCENT1 <= 30 AND PERCENT2 >= 30 THEN LGED30 =
  LOG10(CONC1) + ((30-PERCNT1)/(PERCENT2 - PERCNT1))
  *(LOG10(CONC2) - LOG10(CONC1));

IF PERCENT2 <= 30 AND PERCENT3 >= 30 THEN LGED30 =
  LOG10(CONC2) + ((30-PERCNT2)/(PERCENT3 - PERCNT2))
  *(LOG10(CONC3) - LOG10(CONC2));

IF PERCENT3 <= 30 AND PERCENT4 >= 30 THEN LGED30 =
  LOG10(CONC3) + ((30-PERCNT3)/(PERCENT4 - PERCNT3))
  *(LOG10(CONC4) - LOG10(CONC3));

IF PERCENT4 <= 30 AND PERCENT5 >= 30 THEN LGED30 =
  LOG10(CONC4) + ((30-PERCNT4)/(PERCENT5 - PERCNT4))
  *(LOG10(CONC5) - LOG10(CONC4));

IF PERCENT5 <= 30 AND PERCENT6 >= 30 THEN LGED30 =
  LOG10(CONC5) + ((30-PERCNT5)/(PERCENT6 - PERCNT5))
  *(LOG10(CONC6) - LOG10(CONC5));

IF PERCENT6 <= 30 AND PERCENT7 >= 30 THEN LGED30 =
  LOG10(CONC6) + ((30-PERCNT6)/(PERCENT7 - PERCNT6))
  *(LOG10(CONC7) - LOG10(CONC6));

IF PERCENT7 <= 30 AND PERCENT8 >= 30 THEN LGED30 =
  LOG10(CONC7) + ((30-PERCNT7)/(PERCENT8 - PERCNT7))
  *(LOG10(CONC8) - LOG10(CONC7));

IF PERCENT8 <= 30 AND PERCENT9 >= 30 THEN LGED30 =
  LOG10(CONC8) + ((30-PERCNT8)/(PERCENT9 - PERCNT8))
  *(LOG10(CONC9) - LOG10(CONC8));

IF PERCENT9 <= 30 AND PERCENT10 >= 30 THEN LGED30 =
  LOG10(CONC9) + ((30-PERCNT9)/(PERCENT10 - PERCNT9))
  *(LOG10(CONC10) - LOG10(CONC9));

IF PERCENT10 <= 30 AND PERCENT11 >= 30 THEN LGED30 =
  LOG10(CONC10) + ((30-PERCNT10)/(PERCENT11 - PERCNT10))
  *(LOG10(CONC11) - LOG10(CONC10));

IF PERCENT11 <= 30 AND PERCENT12 >= 30 THEN LGED30 =
  LOG10(CONC11) + ((30-PERCNT11)/(PERCENT12 - PERCNT11))
  *(LOG10(CONC12) - LOG10(CONC11));

IF PERCENT12 <= 30 AND PERCENT13 >= 30 THEN LGED30 =
  LOG10(CONC12) + ((30-PERCNT12)/(PERCENT13 - PERCNT12))
  *(LOG10(CONC13) - LOG10(CONC12));

ED30 = 10**LGED30
NLED30 = - LGED30

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IF PERCENT1 <= 40 AND PERCENT2 >= 40 THEN LGED40 =
  LOG10(CONC1) + ((40-PERCNT1)/(PERCENT2 - PERCNT1))
  *(LOG10(CONC2) - LOG10(CONC1));

IF PERCENT2 <= 40 AND PERCENT3 >= 40 THEN LGED40 =
\[
\begin{align*}
\log_{10}(\text{conc}2 = & \log_{10}(\text{conc}3 - \log_{10}(\text{conc}2)) \\
\text{IF} \quad \text{perc}3 < 40 \quad \text{AND} \quad \text{perc}4 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}3 + ((40 - \text{perc}3) / (\text{perc}4 - \text{perc}3)) \\
& \log_{10}(\text{conc}4 - \log_{10}(\text{conc}3)) \\
\text{IF} \quad \text{perc}4 < 40 \quad \text{AND} \quad \text{perc}5 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}4 + ((40 - \text{perc}4) / (\text{perc}5 - \text{perc}4)) \\
& \log_{10}(\text{conc}5 - \log_{10}(\text{conc}4)) \\
\text{IF} \quad \text{perc}5 < 40 \quad \text{AND} \quad \text{perc}6 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}5 + ((40 - \text{perc}5) / (\text{perc}6 - \text{perc}5)) \\
& \log_{10}(\text{conc}6 - \log_{10}(\text{conc}5)) \\
\text{IF} \quad \text{perc}6 < 40 \quad \text{AND} \quad \text{perc}7 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}6 + ((40 - \text{perc}6) / (\text{perc}7 - \text{perc}6)) \\
& \log_{10}(\text{conc}7 - \log_{10}(\text{conc}6)) \\
\text{IF} \quad \text{perc}7 < 40 \quad \text{AND} \quad \text{perc}8 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}7 + ((40 - \text{perc}7) / (\text{perc}8 - \text{perc}7)) \\
& \log_{10}(\text{conc}8 - \log_{10}(\text{conc}7)) \\
\text{IF} \quad \text{perc}8 < 40 \quad \text{AND} \quad \text{perc}9 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}8 + ((40 - \text{perc}8) / (\text{perc}9 - \text{perc}8)) \\
& \log_{10}(\text{conc}9 - \log_{10}(\text{conc}8)) \\
\text{IF} \quad \text{perc}9 < 40 \quad \text{AND} \quad \text{perc}10 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}9 + ((40 - \text{perc}9) / (\text{perc}10 - \text{perc}9)) \\
& \log_{10}(\text{conc}10 - \log_{10}(\text{conc}9)) \\
\text{IF} \quad \text{perc}10 < 40 \quad \text{AND} \quad \text{perc}11 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}10 + ((40 - \text{perc}10) / (\text{perc}11 - \text{perc}10)) \\
& \log_{10}(\text{conc}11 - \log_{10}(\text{conc}10)) \\
\text{IF} \quad \text{perc}11 < 40 \quad \text{AND} \quad \text{perc}12 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}11 + ((40 - \text{perc}11) / (\text{perc}12 - \text{perc}11)) \\
& \log_{10}(\text{conc}12 - \log_{10}(\text{conc}11)) \\
\text{IF} \quad \text{perc}12 < 40 \quad \text{AND} \quad \text{perc}13 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}12 + ((40 - \text{perc}12) / (\text{perc}13 - \text{perc}12)) \\
& \log_{10}(\text{conc}13 - \log_{10}(\text{conc}12)) \\
\text{IF} \quad \text{perc}13 < 40 \quad \text{AND} \quad \text{perc}14 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}13 + ((40 - \text{perc}13) / (\text{perc}14 - \text{perc}13)) \\
& \log_{10}(\text{conc}14 - \log_{10}(\text{conc}13)) \\
\text{IF} \quad \text{perc}14 < 40 \quad \text{AND} \quad \text{perc}15 > 40 \quad \text{THEN} \quad \text{lgd}40 = \\
& \log_{10}(\text{conc}14 + ((40 - \text{perc}14) / (\text{perc}15 - \text{perc}14)) \\
& \log_{10}(\text{conc}15 - \log_{10}(\text{conc}14)) \\
\text{ed}40 = 10^*\text{lgd}40 \\
\text{nled}40 = -\text{lgd}40 \\
\end{align*}
\]
*(LOG10(CONC5 )-LOG10(CONC4 ));

IF PERCNT5 <=50 AND PERCNT6 >=50 THEN LGED50=
  LOG10(CONC5 )+((50-PERCNT5 )/(PERCNT6 -PERCNT5 ))
  *(LOG10(CONC6 )-LOG10(CONC5 ));

IF PERCNT6 <=50 AND PERCNT7 >=50 THEN LGED50=
  LOG10(CONC6 )+((50-PERCNT6 )/(PERCNT7 -PERCNT6 ))
  *(LOG10(CONC7 )-LOG10(CONC6 ));

IF PERCNT7 <=50 AND PERCNT8 >=50 THEN LGED50=
  LOG10(CONC7 )+((50-PERCNT7 )/(PERCNT8 -PERCNT7 ))
  *(LOG10(CONC8 )-LOG10(CONC7 ));

IF PERCNT8 <=50 AND PERCNT9 >=50 THEN LGED50=
  LOG10(CONC8 )+((50-PERCNT8 )/(PERCNT9 -PERCNT8 ))
  *(LOG10(CONC9 )-LOG10(CONC8 ));

IF PERCNT9 <=50 AND PERCNT10>=50 THEN LGED50=
  LOG10(CONC9 )+((50-PERCNT9 )/(PERCNT10-PERCNT9 ))
  *(LOG10(CONC10)-LOG10(CONC9 ));

IF PERCNT10<=50 AND PERCNT11>=50 THEN LGED50=
  LOG10(CONC10)+((50-PERCNT10 )/(PERCNT11-PERCNT10 ))
  *(LOG10(CONC11)-LOG10(CONC10));

ED50  =  10**LGED50 ;
NLED50 =  - LGED50 ;
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IF PERCNT1<=60 AND PERCNT2 >=60 THEN LGED60=
  LOG10(CONC1 )+((60-PERCNT1 )/(PERCNT2 -PERCNT1 ))
  *(LOG10(CONC2 )-LOG10(CONC1 ));

IF PERCNT2 <=60 AND PERCNT3 >=60 THEN LGED60=
  LOG10(CONC2 )+((60-PERCNT2 )/(PERCNT3 -PERCNT2 ))
  *(LOG10(CONC3 )-LOG10(CONC2 ));

IF PERCNT3 <=60 AND PERCNT4 >=60 THEN LGED60=
  LOG10(CONC3 )+((60-PERCNT3 )/(PERCNT4 -PERCNT3 ))
  *(LOG10(CONC4 )-LOG10(CONC3 ));

IF PERCNT4 <=60 AND PERCNT5 >=60 THEN LGED60=
  LOG10(CONC4 )+((60-PERCNT4 )/(PERCNT5 -PERCNT4 ))
  *(LOG10(CONC5 )-LOG10(CONC4 ));

IF PERCNT5 <=60 AND PERCNT6 >=60 THEN LGED60=
  LOG10(CONC5 )+((60-PERCNT5 )/(PERCNT6 -PERCNT5 ))
  *(LOG10(CONC6 )-LOG10(CONC5 ));

IF PERCNT6 <=60 AND PERCNT7 >=60 THEN LGED60=
  LOG10(CONC6 )+((60-PERCNT6 )/(PERCNT7 -PERCNT6 ))
  *(LOG10(CONC7 )-LOG10(CONC6 ));
IF PERCNT7 <=60 AND PERCNT8 >=60 THEN LGED60 =
  LOG10(CONC7 )+((60-PERCNT7 )/(PERCNT8 -PERCNT7 )
  *(LOG10(CONC8 )-LOG10(CONC7 ));
IF PERCNT8 <=60 AND PERCNT9 >=60 THEN LGED60 =
  LOG10(CONC8 )+((60-PERCNT8 )/(PERCNT9 -PERCNT8 )
  *(LOG10(CONC9 )-LOG10(CONC8 ));
IF PERCNT9 <=60 AND PERCNT10>=60 THEN LGED60 =
  LOG10(CONC9 )+((60-PERCNT9 )/(PERCNT10-PERCNT9 )
  *(LOG10(CONC10)-LOG10(CONC9 ));
IF PERCNT10<60 AND PERCNT11>=60 THEN LGED60 =
  LOG10(CONC10)+((60-PERCNT10)/(PERCNT11-PERCNT10)
  *(LOG10(CONC11)-LOG10(CONC10));
IF PERCNT11<60 AND PERCNT12>=60 THEN LGED60 =
  LOG10(CONC11)+((60-PERCNT11)/(PERCNT12-PERCNT11)
  *(LOG10(CONC12)-LOG10(CONC11));
ED60 = 10**LGED60 ;
NLED60 = - LGED60 ;
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\text{LOG10(CONC9) + ((70 - PERCNT9) / (PERCNT10 - PERCNT9))} \\
* (\text{LOG10(CONC10) - LOG10(CONC9)}) ; \\
\text{IF PERCNT10 < 70 AND PERCNT11 > 70 THEN LGED70 =} \\
\text{LOG10(CONC10) + ((70 - PERCNT10) / (PERCNT11 - PERCNT10))} \\
* (\text{LOG10(CONC11) - LOG10(CONC10)}) ; \\
\text{IF PERCNT11 < 70 AND PERCNT12 > 70 THEN LGED70 =} \\
\text{LOG10(CONC11) + ((70 - PERCNT11) / (PERCNT12 - PERCNT11))} \\
* (\text{LOG10(CONC12) - LOG10(CONC11)}) ; \\
\text{IF PERCNT12 < 70 AND PERCNT13 > 70 THEN LGED70 =} \\
\text{LOG10(CONC12) + ((70 - PERCNT12) / (PERCNT13 - PERCNT12))} \\
* (\text{LOG10(CONC13) - LOG10(CONC12)}) ; \\
\text{IF PERCNT13 < 70 AND PERCNT14 > 70 THEN LGED70 =} \\
\text{LOG10(CONC13) + ((70 - PERCNT13) / (PERCNT14 - PERCNT13))} \\
* (\text{LOG10(CONC14) - LOG10(CONC13)}) ; \\
\text{IF PERCNT14 < 70 AND PERCNT15 > 70 THEN LGED70 =} \\
\text{LOG10(CONC14) + ((70 - PERCNT14) / (PERCNT15 - PERCNT14))} \\
* (\text{LOG10(CONC15) - LOG10(CONC14)}) ; \\
\text{ED70 = 10**LGED70 ;} \\
\text{NLED70 = - LGED70 ;} \\
\]
*(\text{LOG}_1(\text{CONC}_{12})-\text{LOG}_1(\text{CONC}_{11}))

\text{ED}_{80} = 10^{* \text{LGED}_{80}};
\text{NLED}_{80} = - \text{LGED}_{80};

\text{IF PERCNT}_{12} \leq 80 \text{ AND PERCNT}_{13} \geq 80 \text{ THEN } \text{LGED}_{80} =
    \text{LOG}_1(\text{CONC}_{12}) + \frac{(80-\text{PERCNT}_{12})}{(\text{PERCNT}_{13} - \text{PERCNT}_{12})} *
    \text{LOG}_1(\text{CONC}_{13}) - \text{LOG}_1(\text{CONC}_{12});

\text{IF PERCNT}_{13} \leq 80 \text{ AND PERCNT}_{14} \geq 80 \text{ THEN } \text{LGED}_{80} =
    \text{LOG}_1(\text{CONC}_{13}) + \frac{(80-\text{PERCNT}_{13})}{(\text{PERCNT}_{14} - \text{PERCNT}_{13})} *
    \text{LOG}_1(\text{CONC}_{14}) - \text{LOG}_1(\text{CONC}_{13});

\text{ED}_{80} = 10^{* \text{LGED}_{80}};
\text{NLED}_{80} = - \text{LGED}_{80};

\text{IF PERCNT}_{14} \leq 90 \text{ AND PERCNT}_{15} \geq 90 \text{ THEN } \text{LGED}_{90} =
    \text{LOG}_1(\text{CONC}_{14}) + \frac{(90-\text{PERCNT}_{14})}{(\text{PERCNT}_{15} - \text{PERCNT}_{14})} *
    \text{LOG}_1(\text{CONC}_{15}) - \text{LOG}_1(\text{CONC}_{14});

\text{ED}_{90} = 10^{* \text{LGED}_{90}};
\text{NLED}_{90} = - \text{LGED}_{90};
IF PERCNT14<=90 AND PERCNT15>=90 THEN LGED90=
LOG10((CONC14)+(90-PERCNT14)/(PERCNT15-PERCNT14))
*(LOG10(CONC15)-LOG10(CONC14));
ED90 = 10**LGED90 ;
NLED90 = - LGED90 ;

************************************************************
** DATA CONFIGURATION OF ALL1  
**  **
** DATE CHANNEL TISSUE ORDER DRUG R_MAX  
** EFFECT1-EFFECT15 PERCNT1-PERCNT15  
** LGED10-LGED90 ED10-ED90  
**
************************************************************

PROC SORT ; BY DATE CHANNEL ORDER ;
DATA _NULL_ ;
SET ALL1 ; BY DATE CHANNEL ORDER ;
FILE PRINT N=PS NOTITLES ;
L= 6*CHANNEL + 6 ;
IF FIRST.DATE THEN DO ;
PUT PAGE ;
PUT #7 @42 'STATISTICAL ANALYSIS SYSTEM' ;
          @94 DATE DATE9.
          #9 @40 'EFFECT -- MOVEMENT IN MILLIMETERS' ;
END ;
IF FIRST.CHANNEL THEN DO ;
PUT #L @10 '#' ;
       @11 CHANNEL
       @14 '1E-10' @20 '3E-10'
       @26 '1E-09' @32 '3E-09'
       @38 '1E-08' @44 '3E-08'
       @50 '1E-07' @56 '3E-07'
       @62 '1E-06' @68 '3E-06'
       @74 '1E-05' @80 '3E-05'
       @86 '1E-04' @92 '3E-04'
       @98 '1E-03' ;
END ;
PUT ORDER 10
  EFFECT1 14-18 2 EFFECT2 20-24 2
  EFFECT3 26-30 2 EFFECT4 32-36 2
  EFFECT5 38-42 2 EFFECT6 44-48 2
  EFFECT7 50-54 2 EFFECT8 56-60 2
  EFFECT9 62-66 2 EFFECT10 68-72 2
  EFFECT11 74-78 2 EFFECT12 80-84 2
  EFFECT13 85-90 2 EFFECT14 92-96 2
  EFFECT15 98-102 2 ;
IF LAST.DATE THEN DO ;
PUT #52 @1 110*''
DATA _NULL_;  
SET ALL1;  BY DATE CHANNL ORDER;  
FILE PRINT N=PS NOTITLES;  
L=(6 CHANNL + 6);  
IF FIRST.DATE THEN DO;  
PUT _PAGE_;  
PUT #7 @41 'STATISTICAL ANALYSIS SYSTEM'  
#9 @94 DATE DATE9.  
END;  
IF FIRST.CHANNL THEN DO;  
PUT #L @10 '##'  
@11 CHANNL  
@14 '1E-10' @20 '3E-10'  
@26 '1E-09' @32 '3E-09'  
@38 '1E-08' @44 '3E-08'  
@50 '1E-07' @56 '3E-07'  
@62 '1E-06' @68 '3E-06'  
@74 '1E-05' @80 '3E-05'  
@86 '1E-04' @92 '3E-04'  
@98 '1E-03'  
END;  
PUT ORDER 10  
PERCNT1 15-17 PERCNT2 21-23  
PERCNT3 27-29 PERCNT4 33-35  
PERCNT5 39-41 PERCNT6 45-47  
PERCNT7 51-53 PERCNT8 57-59  
PERCNT9 63-65 PERCNT10 69-71  
PERCNT11 75-77 PERCNT12 81-83  
PERCNT13 87-89 PERCNT14 93-95  
PERCNT15 99-101  
END;  
IF LAST.DATE THEN DO;  
PUT #52 @1 110*'_';  
END;  
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *;  
DATA _NULL_;  
SET ALL1;  FILE PRINT N=PS NOTITLES;  
BY DATE CHANNL ORDER;  
L=(6 + 7 CHANNL);  
IF FIRST.DATE THEN DO;  
PUT _PAGE_;  
PUT #7 @71 DATE DATE9.  
#9 @27 'NEGATIVE LOGARITHMS OF ED VALUES';  
END;  
IF FIRST.CHANNL THEN DO;
PUT #L @11 'CHANNEL #'
@21 CHANNL
@28 'ED10'
@34 'ED20'
@40 'ED30'
@46 'ED40'
@52 'ED50'
@58 'ED60'
@64 'ED70'
@70 'ED80'
@76 'ED90';

END;
PUT @9 ORDER
@10 ')
@11 DRUG $15.
@ 11 D R U G  NLED10 28-31 2
NLED20 34-37 2
NLED30 40-43 2
NLED40 46-49 2
NLED50 52-55 2
NLED60 58-61 2
NLED70 64-67 2
NLED80 70-73 2
NLED90 76-79 2

* * * * * * * * * * * * * * * * *
DATA _NULL_
;
SET ALL1;
KEEP DATE CHANNL ORDER DRUG NLED10 NLED20 NLED30 NLED40
NLED50 NLED60 NLED70 NLED80 NLED90;
FORMAT NLED10 3.1 NLED20 3.1 NLED30 3.1 NLED40 3.1
NLED50 3.1 NLED60 3.1 NLED70 3.1 NLED80 3.1 NLED90 3.1;
FILE PRINT N=PS NOTITLES; BY DATE CHANNL ORDER;
   X1 = 111 - ( 10 * NLED10 );
   X2 = 111 - ( 10 * NLED20 );
   X3 = 111 - ( 10 * NLED30 );
   X4 = 111 - ( 10 * NLED40 );
   X5 = 111 - ( 10 * NLED50 );
   X6 = 111 - ( 10 * NLED60 );
   X7 = 111 - ( 10 * NLED70 );
   X8 = 111 - ( 10 * NLED80 );
   X9 = 111 - ( 10 * NLED90 );
   L = ORDER + 5;
IF FIRST.CHANNL THEN DO;
   PUT PAGE;
   PUT #1 @71 DATE DATE9.
   #2 @10 'CHANNEL = '
   @20 CHANNL
   #5 @6 '100% +'
   #6 @11 ']'
   #7 @11 ']';
END ;

PUT #L 16 ORDER
   18 '='
   20 DRUG
#10 @X9 ORDER
#15 @X8 ORDER
#20 @X7 ORDER
#25 @X6 ORDER
#30 @X5 ORDER
#35 @X4 ORDER
#40 @X3 ORDER
#45 @X2 ORDER
#50 @X1 ORDER

* * * * * * * * * * END OF PROGRAM * * * * * * * * * * ;
APPENDIX E

KITSTAT SAS

*****************************************************************************
* * KITSTAT COMPUTES MEANS AND STANDARD ERRORS OF THE * *
* MEANS OF TISSUE RESPONSES AND ED10, ED20 ED30, ETC. * *
* VALUES. FORMAT FOR DATA INPUT IS JUST AS THAT FOR * *
* ITON1. DATA FILES ARE SEPARATE FROM THIS PROGRAM * *
* AND ARE SIMPLY INSERTED JUST FOLLOWING THE CARDS * *
* STATEMENT FOR COMPUTATION. * *
* (FROM $ITSTAT-RICE) * *
* PETER J. RICE * *
* JANUARY 1982 * *
*****************************************************************************

OPTIONS LABEL ERRORABEND NODATE NONUMBER NOSOURCE OVP ;
* ERRORABEND NOSOURCE ;
*****************************************************************************

DATA ALL1 ;
INPUT @1 DATE DATE7. 
@9 CHANNEL 1. 
@11 TISSUE $6. 
@18 ORDER 1. 
  R_MAX 20-24 
@26 DRUG $25.
/

EFFECT3-EFFECT14 ;

EFFECT1 = . ;
EFFECT2 = . ;
EFFECT15 = . ;
PERCNT1 = EFFECT1 / R_MAX * 100 ; CONC1 = 1E-10 ;
PERCNT2 = EFFECT2 / R_MAX * 100 ; CONC2 = 3E-10 ;
PERCNT3 = EFFECT3 / R_MAX * 100 ; CONC3 = 1E-09 ;
PERCNT4 = EFFECT4 / R_MAX * 100 ; CONC4 = 3E-09 ;
PERCNT5 = EFFECT5 / R_MAX * 100 ; CONC5 = 1E-08 ;
PERCNT6 = EFFECT6 / R_MAX * 100 ; CONC6 = 3E-08 ;
PERCNT7 = EFFECT7 / R_MAX * 100 ; CONC7 = 1E-07 ;
PERCNT8 = EFFECT8 / R_MAX * 100 ; CONC8 = 3E-07 ;
PERCNT9 = EFFECT9 / R_MAX * 100 ; CONC9 = 1E-06 ;
PERCNT10 = EFFECT10 / R_MAX * 100 ; CONC10 = 3E-06 ;
PERCNT11 = EFFECT11 / R_MAX * 100 ; CONC11 = 1E-05 ;
PERCNT12 = EFFECT12 / R_MAX * 100 ; CONC12 = 3E-05 ;
PERCNT13 = EFFECT13 / R_MAX * 100 ; CONC13 = 1E-04 ;

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PERCNT14 = EFFECT14 / R_MAX * 100 ; CONC14 = 3E-04 ;
PERCNT15 = EFFECT15 / R_MAX * 100 ; CONC15 = 1E-03 ;
FORM PERCNT1 3.0 PERCNT2 3.0 PERCNT3 3.0 PERCNT4 3.0
PERCNT5 3.0 PERCNT6 3.0 PERCNT7 3.0 PERCNT8 3.0
PERCNT9 3.0 PERCNT10 3.0 PERCNT11 3.0 PERCNT12 3.0
PERCNT13 3.0 PERCNT14 3.0 PERCNT15 3.0 ;
CARDS ;

*****************************************************************************
** DATA SET CONFIGURATION OF ALL1 **
/**
** DATA CHANNEL TISSUE R_MAX DRUG EFFECT1- **
** EFFECT15 PERCNT1-PERCNT15 CONC1-CONC15 **
/**
*****************************************************************************

DATA ALL1 ;
SET ALL1 ;
*** LOGARITHMIC INTERPOLATION TO COMPUTE ED VALUES ***;
OPTIONS NOSOURCE ;
*** **********************************************
IF PERCNT1 <=10 AND PERCNT2 >=10 THEN LGED10=
  LOG10(CONC1 )+((10-PERCNT1 )/(PERCNT2 -PERCNT1 )) *(LOG10(CONC2 )-LOG10(CONC1 ));
IF PERCNT2 <=10 AND PERCNT3 >=10 THEN LGED10=
  LOG10(CONC2 )+((10-PERCNT2 )/(PERCNT3 -PERCNT2 )) *(LOG10(CONC3 )-LOG10(CONC2 ));
IF PERCNT3 <=10 AND PERCNT4 >=10 THEN LGED10=
  LOG10(CONC3 )+((10-PERCNT3 )/(PERCNT4 -PERCNT3 )) *(LOG10(CONC4 )-LOG10(CONC3 ));
IF PERCNT4 <=10 AND PERCNT5 >=10 THEN LGED10=
  LOG10(CONC4 )+((10-PERCNT4 )/(PERCNT5 -PERCNT4 )) *(LOG10(CONC5 )-LOG10(CONC4 ));
IF PERCNT5 <=10 AND PERCNT6 >=10 THEN LGED10=
  LOG10(CONC5 )+((10-PERCNT5 )/(PERCNT6 -PERCNT5 )) *(LOG10(CONC6 )-LOG10(CONC5 ));
IF PERCNT6 <=10 AND PERCNT7 >=10 THEN LGED10=
  LOG10(CONC6 )+((10-PERCNT6 )/(PERCNT7 -PERCNT6 )) *(LOG10(CONC7 )-LOG10(CONC6 ));
IF PERCNT7 <=10 AND PERCNT8 >=10 THEN LGED10=
  LOG10(CONC7 )+((10-PERCNT7 )/(PERCNT8 -PERCNT7 )) *(LOG10(CONC8 )-LOG10(CONC7 ));
IF PERCNT8 <=10 AND PERCNT9 >=10 THEN LGED10=
  LOG10(CONC8 )+((10-PERCNT8 )/(PERCNT9 -PERCNT8 )) *(LOG10(CONC9 )-LOG10(CONC8 ));
IF PERCNT9 <=10 AND PERCNT10 >=10 THEN LGED10=
  LOG10(CONC9 )+((10-PERCNT9 )/(PERCNT10 -PERCNT9 )) *(LOG10(CONC10)-LOG10(CONC9 ));
IF PERCNT10 <=10 AND PERCNT11 >=10 THEN LGED10=
  LOG10(CONC10)+((10-PERCNT10)/(PERCNT11-PERCNT10)) *(LOG10(CONC11)-LOG10(CONC10));
IF PERCNT11 <=10 AND PERCNT12 >=10 THEN LGED10=
  LOG10(CONC11)+((10-PERCNT11)/(PERCNT12-PERCNT11))
* (log10(conc12) - log10(conc11));
if percnt12 <= 10 and percnt13 >= 10 then lg1d10 =
  log10(conc12) + ((10 - percnt12) / (percnt13 - percnt12)) * (log10(conc13) - log10(conc12));
if percnt13 <= 10 and percnt14 >= 10 then lg1d10 =
  log10(conc13) + ((10 - percnt13) / (percnt14 - percnt13)) * (log10(conc14) - log10(conc13));
if percnt14 <= 10 and percnt15 >= 10 then lg1d10 =
  log10(conc14) + ((10 - percnt14) / (percnt15 - percnt14)) * (log10(conc15) - log10(conc14));
ed10 = 10**lg1d10;
nled10 = -lg1d10;
***************
if percnt1 <= 20 and percnt2 >= 20 then lg2d20 =
  log10(conc1) + ((20 - percnt1) / (percnt2 - percnt1)) * (log10(conc2) - log10(conc1));
if percnt2 <= 20 and percnt3 >= 20 then lg2d20 =
  log10(conc2) + ((20 - percnt2) / (percnt3 - percnt2)) * (log10(conc3) - log10(conc2));
if percnt3 <= 20 and percnt4 >= 20 then lg2d20 =
  log10(conc3) + ((20 - percnt3) / (percnt4 - percnt3)) * (log10(conc4) - log10(conc3));
if percnt4 <= 20 and percnt5 >= 20 then lg2d20 =
  log10(conc4) + ((20 - percnt4) / (percnt5 - percnt4)) * (log10(conc5) - log10(conc4));
if percnt5 <= 20 and percnt6 >= 20 then lg2d20 =
  log10(conc5) + ((20 - percnt5) / (percnt6 - percnt5)) * (log10(conc6) - log10(conc5));
if percnt6 <= 20 and percnt7 >= 20 then lg2d20 =
  log10(conc6) + ((20 - percnt6) / (percnt7 - percnt6)) * (log10(conc7) - log10(conc6));
if percnt7 <= 20 and percnt8 >= 20 then lg2d20 =
  log10(conc7) + ((20 - percnt7) / (percnt8 - percnt7)) * (log10(conc8) - log10(conc7));
if percnt8 <= 20 and percnt9 >= 20 then lg2d20 =
  log10(conc8) + ((20 - percnt8) / (percnt9 - percnt8)) * (log10(conc9) - log10(conc8));
if percnt9 <= 20 and percnt10 >= 20 then lg2d20 =
  log10(conc9) + ((20 - percnt9) / (percnt10 - percnt9)) * (log10(conc10) - log10(conc9));
if percnt10 <= 20 and percnt11 >= 20 then lg2d20 =
  log10(conc10) + ((20 - percnt10) / (percnt11 - percnt10)) * (log10(conc11) - log10(conc10));
if percnt11 <= 20 and percnt12 >= 20 then lg2d20 =
  log10(conc11) + ((20 - percnt11) / (percnt12 - percnt11)) * (log10(conc12) - log10(conc11));
if percnt12 <= 20 and percnt13 >= 20 then lg2d20 =
  log10(conc12) + ((20 - percnt12) / (percnt13 - percnt12)) * (log10(conc13) - log10(conc12));
if percnt13 <= 20 and percnt14 >= 20 then lg2d20 =
  log10(conc13) + ((20 - percnt13) / (percnt14 - percnt13)) * (log10(conc14) - log10(conc13));
IF PERCNT14<=20 AND PERCNT15>=20 THEN LGED20 =
\[ \log_{10}(\text{CONC14}) + ((20 - \text{PERCNT14})/(\text{PERCNT15} - \text{PERCNT14})) \times (\log_{10}(\text{CONC15}) - \log_{10}(\text{CONC14})) \];
ED20 = 10**LGED20 ;
NLED20 = - LGED20 ;

* * * * * * * * * * * * * * * * * * * * * * * * * .

IF PERCNT1 <=30 AND PERCNT2 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC1}) + ((30 - \text{PERCNT1})/(\text{PERCNT2} - \text{PERCNT1})) \times (\log_{10}(\text{CONC2}) - \log_{10}(\text{CONC1})) \];
IF PERCNT2 <=30 AND PERCNT3 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC2}) + ((30 - \text{PERCNT2})/(\text{PERCNT3} - \text{PERCNT2})) \times (\log_{10}(\text{CONC3}) - \log_{10}(\text{CONC2})) \];
IF PERCNT3 <=30 AND PERCNT4 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC3}) + ((30 - \text{PERCNT3})/(\text{PERCNT4} - \text{PERCNT3})) \times (\log_{10}(\text{CONC4}) - \log_{10}(\text{CONC3})) \];
IF PERCNT4 <=30 AND PERCNT5 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC4}) + ((30 - \text{PERCNT4})/(\text{PERCNT5} - \text{PERCNT4})) \times (\log_{10}(\text{CONC5}) - \log_{10}(\text{CONC4})) \];
IF PERCNT5 <=30 AND PERCNT6 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC5}) + ((30 - \text{PERCNT5})/(\text{PERCNT6} - \text{PERCNT5})) \times (\log_{10}(\text{CONC6}) - \log_{10}(\text{CONC5})) \];
IF PERCNT6 <=30 AND PERCNT7 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC6}) + ((30 - \text{PERCNT6})/(\text{PERCNT7} - \text{PERCNT6})) \times (\log_{10}(\text{CONC7}) - \log_{10}(\text{CONC6})) \];
IF PERCNT7 <=30 AND PERCNT8 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC7}) + ((30 - \text{PERCNT7})/(\text{PERCNT8} - \text{PERCNT7})) \times (\log_{10}(\text{CONC8}) - \log_{10}(\text{CONC7})) \];
IF PERCNT8 <=30 AND PERCNT9 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC8}) + ((30 - \text{PERCNT8})/(\text{PERCNT9} - \text{PERCNT8})) \times (\log_{10}(\text{CONC9}) - \log_{10}(\text{CONC8})) \];
IF PERCNT9 <=30 AND PERCNT10 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC9}) + ((30 - \text{PERCNT9})/(\text{PERCNT10} - \text{PERCNT9})) \times (\log_{10}(\text{CONC10}) - \log_{10}(\text{CONC9})) \];
IF PERCNT10 <=30 AND PERCNT11 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC10}) + ((30 - \text{PERCNT10})/(\text{PERCNT11} - \text{PERCNT10})) \times (\log_{10}(\text{CONC11}) - \log_{10}(\text{CONC10})) \];
IF PERCNT11 <=30 AND PERCNT12 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC11}) + ((30 - \text{PERCNT11})/(\text{PERCNT12} - \text{PERCNT11})) \times (\log_{10}(\text{CONC12}) - \log_{10}(\text{CONC11})) \];
IF PERCNT12 <=30 AND PERCNT13 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC12}) + ((30 - \text{PERCNT12})/(\text{PERCNT13} - \text{PERCNT12})) \times (\log_{10}(\text{CONC13}) - \log_{10}(\text{CONC12})) \];
IF PERCNT13 <=30 AND PERCNT14 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC13}) + ((30 - \text{PERCNT13})/(\text{PERCNT14} - \text{PERCNT13})) \times (\log_{10}(\text{CONC14}) - \log_{10}(\text{CONC13})) \];
IF PERCNT14 <=30 AND PERCNT15 >=30 THEN LGED30 =
\[ \log_{10}(\text{CONC14}) + ((30 - \text{PERCNT14})/(\text{PERCNT15} - \text{PERCNT14})) \times (\log_{10}(\text{CONC15}) - \log_{10}(\text{CONC14})) \];
ED30 = 10**LGED30 ;
NLED30 = - LGED30 ;

* * * * * * * * * * * * * * * * * * * * * * * * * .

IF PERCNT1 <=40 AND PERCNT2 >=40 THEN LGED40 =

* * * * * * * * * * * * * * * * * * * * * * * * * .
\[ \log_{10}(\text{CONC1}) + \left(\frac{40 - \text{PERCNT1}}{(\text{PERCNT2} - \text{PERCNT1})} \cdot (\log_{10}(\text{CONC2}) - \log_{10}(\text{CONC1}))\right) \]

IF \( \text{PERCNT2} \leq 40 \) AND \( \text{PERCNT3} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC2}) + \left(\frac{40 - \text{PERCNT2}}{(\text{PERCNT3} - \text{PERCNT2})} \cdot (\log_{10}(\text{CONC3}) - \log_{10}(\text{CONC2}))\right) \)

IF \( \text{PERCNT3} \leq 40 \) AND \( \text{PERCNT4} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC3}) + \left(\frac{40 - \text{PERCNT3}}{(\text{PERCNT4} - \text{PERCNT3})} \cdot (\log_{10}(\text{CONC4}) - \log_{10}(\text{CONC3}))\right) \)

IF \( \text{PERCNT4} \leq 40 \) AND \( \text{PERCNT5} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC4}) + \left(\frac{40 - \text{PERCNT4}}{(\text{PERCNT5} - \text{PERCNT4})} \cdot (\log_{10}(\text{CONC5}) - \log_{10}(\text{CONC4}))\right) \)

IF \( \text{PERCNT5} \leq 40 \) AND \( \text{PERCNT6} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC5}) + \left(\frac{40 - \text{PERCNT5}}{(\text{PERCNT6} - \text{PERCNT5})} \cdot (\log_{10}(\text{CONC6}) - \log_{10}(\text{CONC5}))\right) \)

IF \( \text{PERCNT6} \leq 40 \) AND \( \text{PERCNT7} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC6}) + \left(\frac{40 - \text{PERCNT6}}{(\text{PERCNT7} - \text{PERCNT6})} \cdot (\log_{10}(\text{CONC7}) - \log_{10}(\text{CONC6}))\right) \)

IF \( \text{PERCNT7} \leq 40 \) AND \( \text{PERCNT8} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC7}) + \left(\frac{40 - \text{PERCNT7}}{(\text{PERCNT8} - \text{PERCNT7})} \cdot (\log_{10}(\text{CONC8}) - \log_{10}(\text{CONC7}))\right) \)

IF \( \text{PERCNT8} \leq 40 \) AND \( \text{PERCNT9} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC8}) + \left(\frac{40 - \text{PERCNT8}}{(\text{PERCNT9} - \text{PERCNT8})} \cdot (\log_{10}(\text{CONC9}) - \log_{10}(\text{CONC8}))\right) \)

IF \( \text{PERCNT9} \leq 40 \) AND \( \text{PERCNT10} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC9}) + \left(\frac{40 - \text{PERCNT9}}{(\text{PERCNT10} - \text{PERCNT9})} \cdot (\log_{10}(\text{CONC10}) - \log_{10}(\text{CONC9}))\right) \)

IF \( \text{PERCNT10} \leq 40 \) AND \( \text{PERCNT11} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC10}) + \left(\frac{40 - \text{PERCNT10}}{(\text{PERCNT11} - \text{PERCNT10})} \cdot (\log_{10}(\text{CONC11}) - \log_{10}(\text{CONC10}))\right) \)

IF \( \text{PERCNT11} \leq 40 \) AND \( \text{PERCNT12} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC11}) + \left(\frac{40 - \text{PERCNT11}}{(\text{PERCNT12} - \text{PERCNT11})} \cdot (\log_{10}(\text{CONC12}) - \log_{10}(\text{CONC11}))\right) \)

IF \( \text{PERCNT12} \leq 40 \) AND \( \text{PERCNT13} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC12}) + \left(\frac{40 - \text{PERCNT12}}{(\text{PERCNT13} - \text{PERCNT12})} \cdot (\log_{10}(\text{CONC13}) - \log_{10}(\text{CONC12}))\right) \)

IF \( \text{PERCNT13} \leq 40 \) AND \( \text{PERCNT14} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC13}) + \left(\frac{40 - \text{PERCNT13}}{(\text{PERCNT14} - \text{PERCNT13})} \cdot (\log_{10}(\text{CONC14}) - \log_{10}(\text{CONC13}))\right) \)

IF \( \text{PERCNT14} \leq 40 \) AND \( \text{PERCNT15} \geq 40 \) THEN \( \text{LGED40} = \log_{10}(\text{CONC14}) + \left(\frac{40 - \text{PERCNT14}}{(\text{PERCNT15} - \text{PERCNT14})} \cdot (\log_{10}(\text{CONC15}) - \log_{10}(\text{CONC14}))\right) \)

ED40 = 10**(LGED40)
NLED40 = - LGED40

**********

IF \( \text{PERCNT1} \leq 50 \) AND \( \text{PERCNT2} \geq 50 \) THEN \( \text{LGED50} = \log_{10}(\text{CONC1}) + \left(\frac{50 - \text{PERCNT1}}{(\text{PERCNT2} - \text{PERCNT1})} \cdot (\log_{10}(\text{CONC2}) - \log_{10}(\text{CONC1}))\right) \)

IF \( \text{PERCNT2} \leq 50 \) AND \( \text{PERCNT3} \geq 50 \) THEN \( \text{LGED50} = \log_{10}(\text{CONC2}) + \left(\frac{50 - \text{PERCNT2}}{(\text{PERCNT3} - \text{PERCNT2})} \cdot (\log_{10}(\text{CONC3}) - \log_{10}(\text{CONC2}))\right) \)

IF \( \text{PERCNT3} \leq 50 \) AND \( \text{PERCNT4} \geq 50 \) THEN \( \text{LGED50} = \log_{10}(\text{CONC3}) + \left(\frac{50 - \text{PERCNT3}}{(\text{PERCNT4} - \text{PERCNT3})} \cdot (\log_{10}(\text{CONC4}) - \log_{10}(\text{CONC3}))\right) \)
*(\text{LOG}10(\text{CONC}4) - \text{LOG}10(\text{CONC}3)) ; \\
\text{IF PERCNT4} \leq 50 \text{ AND PERCNT5} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}4) + ((50-\text{PERCENT}4)/(\text{PERCENT}5 - \text{PERCENT}4)) * \text{LOG}10(\text{CONC}5) - \text{LOG}10(\text{CONC}4) ; \\
\text{IF PERCNT5} \leq 50 \text{ AND PERCNT6} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}5) + ((50-\text{PERCENT}5)/(\text{PERCENT}6 - \text{PERCENT}5)) * \text{LOG}10(\text{CONC}6) - \text{LOG}10(\text{CONC}5) ; \\
\text{IF PERCNT6} \leq 50 \text{ AND PERCNT7} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}6) + ((50-\text{PERCENT}6)/(\text{PERCENT}7 - \text{PERCENT}6)) * \text{LOG}10(\text{CONC}7) - \text{LOG}10(\text{CONC}6) ; \\
\text{IF PERCNT7} \leq 50 \text{ AND PERCNT8} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}7) + ((50-\text{PERCENT}7)/(\text{PERCENT}8 - \text{PERCENT}7)) * \text{LOG}10(\text{CONC}8) - \text{LOG}10(\text{CONC}7) ; \\
\text{IF PERCNT8} \leq 50 \text{ AND PERCNT9} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}8) + ((50-\text{PERCENT}8)/(\text{PERCENT}9 - \text{PERCENT}8)) * \text{LOG}10(\text{CONC}9) - \text{LOG}10(\text{CONC}8) ; \\
\text{IF PERCNT9} \leq 50 \text{ AND PERCENT10} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}9) + ((50-\text{PERCENT}9)/(\text{PERCENT}10 - \text{PERCENT}9)) * \text{LOG}10(\text{CONC}10) - \text{LOG}10(\text{CONC}9) ; \\
\text{IF PERCENT10} \leq 50 \text{ AND PERCENT11} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}10) + ((50-\text{PERCENT}10)/(\text{PERCENT}11 - \text{PERCENT}10)) * \text{LOG}10(\text{CONC}11) - \text{LOG}10(\text{CONC}10) ; \\
\text{IF PERCENT11} \leq 50 \text{ AND PERCENT12} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}11) + ((50-\text{PERCENT}11)/(\text{PERCENT}12 - \text{PERCENT}11)) * \text{LOG}10(\text{CONC}12) - \text{LOG}10(\text{CONC}11) ; \\
\text{IF PERCENT12} \leq 50 \text{ AND PERCENT13} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}12) + ((50-\text{PERCENT}12)/(\text{PERCENT}13 - \text{PERCENT}12)) * \text{LOG}10(\text{CONC}13) - \text{LOG}10(\text{CONC}12) ; \\
\text{IF PERCENT13} \leq 50 \text{ AND PERCENT14} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}13) + ((50-\text{PERCENT}13)/(\text{PERCENT}14 - \text{PERCENT}13)) * \text{LOG}10(\text{CONC}14) - \text{LOG}10(\text{CONC}13) ; \\
\text{IF PERCENT14} \leq 50 \text{ AND PERCENT15} \geq 50 \text{ THEN LGED50} = \text{LOG}10(\text{CONC}14) + ((50-\text{PERCENT}14)/(\text{PERCENT}15 - \text{PERCENT}14)) * \text{LOG}10(\text{CONC}15) - \text{LOG}10(\text{CONC}14) ; \\
\text{ED50} = 10^{\text{LGED50}} ; \\
\text{NLED50} = - \text{LGED50} ; \\
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
IF PERCNT6 <= 60 AND PERCNT7 >= 60 THEN LGED60 =
   LOG10(CONC6) +((60-PERCNT6 )/(PERCNT7 -PERCNT6 ))
   *(LOG10(CONC7 )-LOG10(CONC6 ));
IF PERCNT7 <= 60 AND PERCNT8 >= 60 THEN LGED60 =
   LOG10(CONC7) +((60-PERCNT7 )/(PERCNT8 -PERCNT7 ))
   *(LOG10(CONC8 )-LOG10(CONC7 ));
IF PERCNT8 <= 60 AND PERCNT9 >= 60 THEN LGED60 =
   LOG10(CONC8 )+((60-PERCNT8 )/(PERCNT9 -PERCNT8 ))
   *(LOG10(CONC9 )-LOG10(CONC8 ));
IF PERCNT9 <= 60 AND PERCNT10 >= 60 THEN LGED60 =
   LOG10(CONC9 )+((60-PERCNT9 )/(PERCNT10-PERCNT9 ))
   *(LOG10(CONC10)-LOG10(CONC9 ));
IF PERCNT10<=60 AND PERCNT11>=60 THEN LGED60 =
   LOG10(CONC10)+(60-PERCNT10 )/(PERCNT11-PERCNT10 )
   *(LOG10(CONC11)-LOG10(CONC10));
IF PERCNT11<=60 AND PERCNT12>=60 THEN LGED60 =
   LOG10(CONC11)+((60-PERCNT11 )/(PERCNT12-PERCNT11 ))
   *(LOG10(CONC12)-LOG10(CONC11));
IF PERCNT12<=60 AND PERCNT13>=60 THEN LGED60 =
   LOG10(CONC12)+((60-PERCNT12 )/(PERCNT13-PERCNT12 ))
   *(LOG10(CONC13)-LOG10(CONC12));
IF PERCNT13<=60 AND PERCNT14>=60 THEN LGED60 =
   LOG10(CONC13)+((60-PERCNT13 )/(PERCNT14-PERCNT13 ))
   *(LOG10(CONC14)-LOG10(CONC13));
IF PERCNT14<=60 AND PERCNT15>=60 THEN LGED60 =
   LOG10(CONC14)+((60-PERCNT14 )/(PERCNT15-PERCNT14 ))
   *(LOG10(CONC15)-LOG10(CONC14));
ED60 = 10**LGED60 ;
NLED60 = -LGED60 ;

IF PERCNT1<=70 AND PERCNT2 >= 70 THEN LGED70 =
   LOG10(CONC1 )+((70-PERCNT1 )/(PERCNT2 -PERCNT1 ))
   *(LOG10(CONC2 )-LOG10(CONC1 ));
IF PERCNT2 <= 70 AND PERCNT3 >= 70 THEN LGED70 =
   LOG10(CONC2)+((70-PERCNT2 )/(PERCNT3 -PERCNT2 ))
   *(LOG10(CONC3 )-LOG10(CONC2 ));
IF PERCNT3 <= 70 AND PERCNT4 >= 70 THEN LGED70 =
   LOG10(CONC3 )+((70-PERCNT3 )/(PERCNT4 -PERCNT3 ))
   *(LOG10(CONC4 )-LOG10(CONC3 ));
IF PERCNT4 <= 70 AND PERCNT5 >= 70 THEN LGED70 =
   LOG10(CONC4 )+((70-PERCNT4 )/(PERCNT5 -PERCNT4 ))
   *(LOG10(CONC5 )-LOG10(CONC4 ));
IF PERCNT5 <= 70 AND PERCNT6 >= 70 THEN LGED70 =
   LOG10(CONC5 )+((70-PERCNT5 )/(PERCNT6 -PERCNT5 ))
   *(LOG10(CONC6 )-LOG10(CONC5 ));
IF PERCNT6 <= 70 AND PERCNT7 >= 70 THEN LGED70 =
   LOG10(CONC6 )+((70-PERCNT6 )/(PERCNT7 -PERCNT6 ))
   *(LOG10(CONC7 )-LOG10(CONC6 ));
IF PERCNT7 <= 70 AND PERCNT8 >= 70 THEN LGED70 =
   LOG10(CONC7 )+((70-PERCNT7 )/(PERCNT8 -PERCNT7 ))
   *(LOG10(CONC8 )-LOG10(CONC7 ));
IF PERCNT8 <= 70 AND PERCNT9 >= 70 THEN LGED70 =
\[ L_{0G}(\text{conc}9) + \left( \frac{(70-\text{perc}9)}{(\text{perc}10-\text{perc}9} \right) \times \left( L_{0G}(\text{conc}10) - L_{0G}(\text{conc}9) \right) \]

**IF** \( \text{perc}9 \leq 70 \) *AND* \( \text{perc}10 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}9) + \left( \frac{(70-\text{perc}9)}{(\text{perc}10-\text{perc}9} \right) \times \left( L_{0G}(\text{conc}10) - L_{0G}(\text{conc}9) \right) \)

**IF** \( \text{perc}10 \leq 70 \) *AND* \( \text{perc}11 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}10) + \left( \frac{(70-\text{perc}10)}{(\text{perc}11-\text{perc}10} \right) \times \left( L_{0G}(\text{conc}11) - L_{0G}(\text{conc}10) \right) \)

**IF** \( \text{perc}11 \leq 70 \) *AND* \( \text{perc}12 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}11) + \left( \frac{(70-\text{perc}11)}{(\text{perc}12-\text{perc}11} \right) \times \left( L_{0G}(\text{conc}12) - L_{0G}(\text{conc}11) \right) \)

**IF** \( \text{perc}12 \leq 70 \) *AND* \( \text{perc}13 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}12) + \left( \frac{(70-\text{perc}12)}{(\text{perc}13-\text{perc}12} \right) \times \left( L_{0G}(\text{conc}13) - L_{0G}(\text{conc}12) \right) \)

**IF** \( \text{perc}13 \leq 70 \) *AND* \( \text{perc}14 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}13) + \left( \frac{(70-\text{perc}13)}{(\text{perc}14-\text{perc}13} \right) \times \left( L_{0G}(\text{conc}14) - L_{0G}(\text{conc}13) \right) \)

**IF** \( \text{perc}14 \leq 70 \) *AND* \( \text{perc}15 \geq 70 \) **THEN**  \( L_{0G}70 = L_{0G}(\text{conc}14) + \left( \frac{(70-\text{perc}14)}{(\text{perc}15-\text{perc}14} \right) \times \left( L_{0G}(\text{conc}15) - L_{0G}(\text{conc}14) \right) \)

**ED70 = 10**\( L_{0G}70 \);

**NLED70 = - L_{0G}70**;

**IF** \( \text{perc}1 \leq 80 \) *AND* \( \text{perc}2 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}1) + \left( \frac{(80-\text{perc}1)}{(\text{perc}2-\text{perc}1} \right) \times \left( L_{0G}(\text{conc}2) - L_{0G}(\text{conc}1) \right) \)

**IF** \( \text{perc}2 \leq 80 \) *AND* \( \text{perc}3 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}2) + \left( \frac{(80-\text{perc}2)}{(\text{perc}3-\text{perc}2} \right) \times \left( L_{0G}(\text{conc}3) - L_{0G}(\text{conc}2) \right) \)

**IF** \( \text{perc}3 \leq 80 \) *AND* \( \text{perc}4 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}3) + \left( \frac{(80-\text{perc}3)}{(\text{perc}4-\text{perc}3} \right) \times \left( L_{0G}(\text{conc}4) - L_{0G}(\text{conc}3) \right) \)

**IF** \( \text{perc}4 \leq 80 \) *AND* \( \text{perc}5 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}4) + \left( \frac{(80-\text{perc}4)}{(\text{perc}5-\text{perc}4} \right) \times \left( L_{0G}(\text{conc}5) - L_{0G}(\text{conc}4) \right) \)

**IF** \( \text{perc}5 \leq 80 \) *AND* \( \text{perc}6 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}5) + \left( \frac{(80-\text{perc}5)}{(\text{perc}6-\text{perc}5} \right) \times \left( L_{0G}(\text{conc}6) - L_{0G}(\text{conc}5) \right) \)

**IF** \( \text{perc}6 \leq 80 \) *AND* \( \text{perc}7 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}6) + \left( \frac{(80-\text{perc}6)}{(\text{perc}7-\text{perc}6} \right) \times \left( L_{0G}(\text{conc}7) - L_{0G}(\text{conc}6) \right) \)

**IF** \( \text{perc}7 \leq 80 \) *AND* \( \text{perc}8 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}7) + \left( \frac{(80-\text{perc}7)}{(\text{perc}8-\text{perc}7} \right) \times \left( L_{0G}(\text{conc}8) - L_{0G}(\text{conc}7) \right) \)

**IF** \( \text{perc}8 \leq 80 \) *AND* \( \text{perc}9 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}8) + \left( \frac{(80-\text{perc}8)}{(\text{perc}9-\text{perc}8} \right) \times \left( L_{0G}(\text{conc}9) - L_{0G}(\text{conc}8) \right) \)

**IF** \( \text{perc}9 \leq 80 \) *AND* \( \text{perc}10 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}9) + \left( \frac{(80-\text{perc}9)}{(\text{perc}10-\text{perc}9} \right) \times \left( L_{0G}(\text{conc}10) - L_{0G}(\text{conc}9) \right) \)

**IF** \( \text{perc}10 \leq 80 \) *AND* \( \text{perc}11 \geq 80 \) **THEN**  \( L_{0G}80 = L_{0G}(\text{conc}10) + \left( \frac{(80-\text{perc}10)}{(\text{perc}11-\text{perc}10} \right) \times \left( L_{0G}(\text{conc}11) - L_{0G}(\text{conc}10) \right) \)
*(LOG10(CONC11)-LOG10(CONC10)) ;
IF PERCNT11<=80 AND PERCNT12>=80 THEN LGED80=
    *(LOG10(CONC11)+((80-PERCNT11)/(PERCNT12-PERCNT11))
    *(LOG10(CONC12)-LOG10(CONC11)) ;
IF PERCNT12<=80 AND PERCNT13>=80 THEN LGED80=
    *(LOG10(CONC12)+((80-PERCNT12)/(PERCNT13-PERCNT12))
    *(LOG10(CONC13)-LOG10(CONC12)) ;
IF PERCNT13<=80 AND PERCNT14>=80 THEN LGED80=
    *(LOG10(CONC13)+((80-PERCNT13)/(PERCNT14-PERCNT13))
    *(LOG10(CONC14)-LOG10(CONC13)) ;
IF PERCNT14<=80 AND PERCNT15>=80 THEN LGED80=
    *(LOG10(CONC15)-LOG10(CONC14)) ;
ED80 = 10**LGED80 ;
NLED80 = - LGED80 ;
** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
IF PERCNT1<=90 AND PERCNT2 >=90 THEN LGED90=
    *(LOG10(CONC1)+((90-PERCNT1)/(PERCNT2-PERCNT1))
    *(LOG10(CONC2)-LOG10(CONC1)) ;
IF PERCNT2 <=90 AND PERCNT3 >=90 THEN LGED90=
    *(LOG10(CONC2)+((90-PERCNT2)/(PERCNT3-PERCNT2))
    *(LOG10(CONC3)-LOG10(CONC2)) ;
IF PERCNT3 <=90 AND PERCNT4 >=90 THEN LGED90=
    *(LOG10(CONC3)+((90-PERCNT3)/(PERCNT4-PERCNT3))
    *(LOG10(CONC4)-LOG10(CONC3)) ;
IF PERCNT4 <=90 AND PERCNT5 >=90 THEN LGED90=
    *(LOG10(CONC4)+((90-PERCNT4)/(PERCNT5-PERCNT4))
    *(LOG10(CONC5)-LOG10(CONC4)) ;
IF PERCNT5 <=90 AND PERCNT6 >=90 THEN LGED90=
    *(LOG10(CONC5)+((90-PERCNT5)/(PERCNT6-PERCNT5))
    *(LOG10(CONC6)-LOG10(CONC5)) ;
IF PERCNT6 <=90 AND PERCNT7 >=90 THEN LGED90=
    *(LOG10(CONC6)+((90-PERCNT6)/(PERCNT7-PERCNT6))
    *(LOG10(CONC7)-LOG10(CONC6)) ;
IF PERCNT7 <=90 AND PERCNT8 >=90 THEN LGED90=
    *(LOG10(CONC7)+((90-PERCNT7)/(PERCNT8-PERCNT7))
    *(LOG10(CONC8)-LOG10(CONC7)) ;
IF PERCNT8 <=90 AND PERCNT9 >=90 THEN LGED90=
    *(LOG10(CONC8)+((90-PERCNT8)/(PERCNT9-PERCNT8))
    *(LOG10(CONC9)-LOG10(CONC8)) ;
IF PERCNT9 <=90 AND PERCNT10>=90 THEN LGED90=
    *(LOG10(CONC9)+((90-PERCNT9)/(PERCNT10-PERCNT9))
    *(LOG10(CONC10)-LOG10(CONC9)) ;
IF PERCNT10<=90 AND PERCNT11>=90 THEN LGED90=
    *(LOG10(CONC10)+((90-PERCNT10)/(PERCNT11-PERCNT10))
    *(LOG10(CONC11)-LOG10(CONC10)) ;
IF PERCNT11<=90 AND PERCNT12>=90 THEN LGED90=
    *(LOG10(CONC11)+((90-PERCNT11)/(PERCNT12-PERCNT11))
    *(LOG10(CONC12)-LOG10(CONC11)) ;
IF PERCNT12<=90 AND PERCNT13>=90 THEN LGED90=
    *(LOG10(CONC12)+((90-PERCNT12)/(PERCNT13-PERCNT12))
    *(LOG10(CONC13)-LOG10(CONC12)) ;
IF PERCNT13<=90 AND PERCNT14>=90 THEN LGED90 =
   LOG10(CONC13) + ((90-PERCNT13)/(PERCNT14-PERCNT13)) )
   *(LOG10(CONC14)-LOG10(CONC13)) ;
IF PERCNT14<=90 AND PERCNT15>=90 THEN LGED90 =
   LOG10(CONC14) + ((90-PERCNT14)/(PERCNT15-PERCNT14))
   *(LOG10(CONC15)-LOG10(CONC14)) ;
ED90 = 10**LGED90 ;
NLED90 = - LGED90 ;

OPTIONS NOSOURCE ;
*****************************************************************************
** DATA CONFIGURATION OF ALL1 **
**
** DATE CHANNEL TISSUE ORDER DRUG R_MAX **
** EFFECT1-EFFECT15 PERCNT1-PERCNT15 **
** LGED10-LGED90 ED10-ED90 **
**
*****************************************************************************

***********************************************************************
***** SECTION EIGHT OF OUTPUT - STATISTICS: *****
PERCENT OF MAXIMAL CONTRACTION BY DRUG / CONCENTRATION *****
*****************************************************************************

PROC SORT DATA=ALL1 ; BY DRUG ;
PROC MEANS DATA=ALL1 NOPRINT
   MEAN VAR STD STDERR KURTOSIS SKEWNESS N ; BY DRUG ;
   VARIABLES PERCNT1-PERCNT15 ;
   OUTPUT OUT=STAT6 MEAN=M1-M15 VAR=V1-V15 STD=SD1-SD15
      STDERR=SE1-SE15 KURTOSIS=K1-K15 SKEWNESS=S1-S15 N=N1-N15 ;
DATA STAT6 ;
SET STAT6 ;
   DROP M1-M15 V1-V15 SD1-SD15 SE1-SE15
      K1-K15 S1-S15 N1-N15 ;
   MEAN=M1 ;
   VARIANCE=V1 ;
   STD_DEV=SD1 ;
   STDERR=SE1 ;
   KURTOSIS=K1 ;
   SKEWNESS=S1 ;
   N=N1 ;
   CONC=1E-10 ;
   SE_UP = M1 + SE1 ;
   SE_LO = M1 - SE1 ;
   OUTPUT ;
   MEAN=M2 ;
   VARIANCE=V2 ;
   STD_DEV=SD2 ;
   STDERR=SE2 ;
   KURTOSIS=K2 ;
   SKEWNESS=S2 ;
   N=N2 ;
   CONC=3E-10 ;
   SE_UP = M2 + SE2 ;
   SE_LO = M2 - SE2 ;
   OUTPUT ;
   MEAN=M3 ;
   VARIANCE=V3 ;
   STD_DEV=SD3 ;
   STDERR=SE3 ;
   KURTOSIS=K3 ;
   SKEWNESS=S3 ;
   N=N3 ;
   CONC=1E-09 ;
   SE_UP = M3 + SE3 ;
   SE_LO = M3 - SE3 ;
   OUTPUT ;
   MEAN=M4 ;
   VARIANCE=V4 ;
   STD_DEV=SD4 ;
   STDERR=SE4 ;
   KURTOSIS=K4 ;
   SKEWNESS=S4 ;
   N=N4 ;
   CONC=3E-09 ;
   SE_UP = M4 + SE4 ;
   SE_LO = M4 - SE4 ;
   OUTPUT ;
   MEAN=M5 ;
   VARIANCE=V5 ;
   STD_DEV=SD5 ;
   STDERR=SE5 ;
   KURTOSIS=K5 ;
   SKEWNESS=S5 ;
   N=N5 ;
   CONC=1E-08 ;
   SE_UP = M5 + SE5 ;
   SE_LO = M5 - SE5 ;
   OUTPUT ;
MEAN=M6; VARIANCE=V6; STD_DEV=SD6; STDERR=SE6;
KURTOSIS=K6; SKEWNESS=S6; N=N6; CONC=3E-08;
SE_UP = M6 + SE6; SE_LO = M6 - SE6;
OUTPUT;
MEAN=M7; VARIANCE=V7; STD_DEV=SD7; STDERR=SE7;
KURTOSIS=K7; SKEWNESS=S7; N=N7; CONC=1E-07;
SE_UP = M7 + SE7; SE_LO = M7 - SE7;
OUTPUT;
MEAN=M8; VARIANCE=V8; STD_DEV=SD8; STDERR=SE8;
KURTOSIS=K8; SKEWNESS=S8; N=N8; CONC=3E-07;
SE_UP = M8 + SE8; SE_LO = M8 - SE8;
OUTPUT;
MEAN=M9; VARIANCE=V9; STD_DEV=SD9; STDERR=SE9;
KURTOSIS=K9; SKEWNESS=S9; N=N9; CONC=1E-06;
SE_UP = M9 + SE9; SE_LO = M9 - SE9;
OUTPUT;
MEAN=M10; VARIANCE=V10; STD_DEV=SD10; STDERR=SE10;
KURTOSIS=K10; SKEWNESS=S10; N=N10; CONC=3E-05;
SE_UP = M10 + SE10; SE_LO = M10 - SE10;
OUTPUT;
MEAN=M11; VARIANCE=V11; STD_DEV=SD11; STDERR=SE11;
KURTOSIS=K11; SKEWNESS=S11; N=N11; CONC=1E-05;
SE_UP = M11 + SE11; SE_LO = M11 - SE11;
OUTPUT;
MEAN=M12; VARIANCE=V12; STD_DEV=SD12; STDERR=SE12;
KURTOSIS=K12; SKEWNESS=S12; N=N12; CONC=3E-05;
SE_UP = M12 + SE12; SE_LO = M12 - SE12;
OUTPUT;
MEAN=M13; VARIANCE=V13; STD_DEV=SD13; STDERR=SE13;
KURTOSIS=K13; SKEWNESS=S13; N=N13; CONC=1E-04;
SE_UP = M13 + SE13; SE_LO = M13 - SE13;
OUTPUT;
MEAN=M14; VARIANCE=V14; STD_DEV=SD14; STDERR=SE14;
KURTOSIS=K14; SKEWNESS=S14; N=N14; CONC=3E-04;
SE_UP = M14 + SE14; SE_LO = M14 - SE14;
OUTPUT;
MEAN=M15; VARIANCE=V15; STD_DEV=SD15; STDERR=SE15;
KURTOSIS=K15; SKEWNESS=S15; N=N15; CONC=1E-03;
SE_UP = M15 + SE15; SE_LO = M15 - SE15;
OUTPUT;
FORMAT CONC EB8.1 MEAN 7.3 VARIANCE 6.3 STD_DEV 6.3 STDERR 6.3;
PROC PRINT DATA=STAT6 DOUBLE PAGE ; BY DRUG ;
TITLE3 STATISTICS : PERCENT OF MAXIMAL KCL EFFECT ;
TITLE5 TISSUE : F344 RAT BLADDER ;
VARIABLES MEAN STDERR STD_DEV N VARIANCE SKEWNESS KURTOSIS ;
FORMAT MEAN 6.2 STDERR 6.2 STD_DEV 6.2 VARIANCE 6.2 SKEWNESS 4.2 KURTOSIS 4.2 ;
ID CONC ;
PROC PRINT DATA=STAT6 ; BY DRUG ;
TITLE3 STATISTICS FOR PLOTTING : PERCENT OF MAXIMAL KCL EFFECT ;
TITLE5 TISSUE : F344 RAT BLADDER ;
VARIABLES MEAN STDERR SE_UP SE_LO N;
FORMAT MEAN 5.1 STDERR 5.1 SE_UP 5.1 SE_LO 5.1;
ID CONC;

*** SECTION TEN OF OUTPUT -- LOG OF ED VALUES BY DRUG **;
PROC MEANS DATA=AL1 NOPRINT MAXDEC=2;
   MEAN VAR STD STDERR KURTOSIS SKEWNESS N;
   BY DRUG;
   VARIABLES NLED10 NLED20 NLED30 NLED40 NLED50;
   NLED60 NLED70 NLED80 NLED90;
   OUTPUT OUT=STAT5 MEAN=M1-M9 VAR=V1-V9 STD=SD1-SD9;
   STDERR=SE1-SE9 KURTOSIS=K1-K9 SKEWNESS=S1-S9;
   N=N1-N9;
DATA STAT5;
SET STAT5;
DROP M1-M9 V1-V9 SD1-SD9 SE1-SE9;
MEAN=M1;
VARIANCE=V1;
STD_DEV=SD1;
STDERR=SE1;
KURTOSIS=K1;
SKEWNESS=S1;
N=N1;
EFFECT=0.10;
SE_UP = M1 + SE1;
SE_LO = M1 - SE1;
OUTPUT;
MEAN=M2;
VARIANCE=V2;
STD_DEV=SD2;
STDERR=SE2;
KURTOSIS=K2;
SKEWNESS=S2;
N=N2;
EFFECT=0.20;
SE_UP = M2 + SE2;
SE_LO = M2 - SE2;
OUTPUT;
MEAN=M3;
VARIANCE=V3;
STD_DEV=SD3;
STDERR=SE3;
KURTOSIS=K3;
SKEWNESS=S3;
N=N3;
EFFECT=0.30;
SE_UP = M3 + SE3;
SE_LO = M3 - SE3;
OUTPUT;
MEAN=M4;
VARIANCE=V4;
STD_DEV=SD4;
STDERR=SE4;
KURTOSIS=K4;
SKEWNESS=S4;
N=N4;
EFFECT=0.40;
SE_UP = M4 + SE4;
SE_LO = M4 - SE4;
OUTPUT;
MEAN=M5;
VARIANCE=V5;
STD_DEV=SD5;
STDERR=SE5;
KURTOSIS=K5;
SKEWNESS=S5;
N=N5;
EFFECT=0.50;
SE_UP = M5 + SE5;
SE_LO = M5 - SE5;
OUTPUT;
MEAN=M6;
VARIANCE=V6;
STD_DEV=SD6;
STDERR=SE6;
KURTOSIS=K6;
SKEWNESS=S6;
N=N6;
EFFECT=0.60;
SE_UP = M6 + SE6;
SE_LO = M6 - SE6;
OUTPUT;
MEAN=M7;
VARIANCE=V7;
STD_DEV=SD7;
STDERR=SE7;
KURTOSIS=K7;
SKEWNESS=S7;
N=N7;
EFFECT=0.70;
SE_UP = M7 + SE7;
SE_LO = M7 - SE7;
OUTPUT;
MEAN=M8;
VARIANCE=V8;
STD_DEV=SD8;
STDERR=SE8;
KURTOSIS=K8;
SKEWNESS=S8;
N=N8;
EFFECT=0.80;
SE_UP = M8 + SE8;
SE_LO = M8 - SE8;
OUTPUT;
MEAN=M9;
VARIANCE=V9;
STD_DEV=SD9;
STDERR=SE9;
KURTOSIS=K9;
SKEWNESS=S9;
N=N9;
EFFECT=0.90;
SE_UP = M9 + SE9;
SE_LO = M9 - SE9;
OUTPUT;
FORMAT EFFECT 4.2 MEAN 5.3 VARIANCE 5.3 STD_DEV 5.3 STDERR 5.3;
KURTOSIS 5.3 SKEWNESS 5.3 N 3.0;
DATA STAT5 ; SET STAT5 ; IF N > 0 ;
PROC SORT DATA=STAT5 ; BY DRUG ;
PROC PRINT DATA=STAT5 DOUBLE PAGE ; BY DRUG ;
  TITLE3 STATISTICS : NEGATIVE LOGARITHMS OF ED VALUES ;
TITLE5 TISSUE : F344 RAT BLADDER ;
  VARIABLES MEAN STDERR STD DEV N VARIANCE SKEWNESS KURTOSIS ;
  FORMAT MEAN 5.3 STDERR 5.3 STD_DEV 5.3 VARIANCE 5.2
             SKEWNESS 4.2 KURTOSIS 4.2 ;
  ID EFFECT ;
PROC PRINT DATA=STAT5 ; BY DRUG ;
  TITLE3 STATISTICS : NEGATIVE LOGARITHMS OF ED VALUES ;
TITLE5 TISSUE : F344 RAT BLADDER ;
  VARIABLES MEAN STDERR SE_UP SE_LO N ;
  FORMAT MEAN 4.2 STDERR 4.2 SE_UP 4.2 SE_LO 4.2 ;
  ID EFFECT ;
****************************** END OF PROGRAM ****************************** ;
APPENDIX F

SCATCHARD SAS

******************************************************************************;
*  *
* SCATCHARD COMPUTES BOUND, FREE, AND BOUND/FREE FROM RAW. *
* BINDING DATA. SCATCHARD PLOTS BOUND VS FREE (SATURATION CURVE) AND BOUND/FREE VS. BOUND (SCATCHARD ANALYSIS).
* AN EXAMPLE OF DATA IN THE PROPER FORMAT FOR INPUT APPEARS *
* APPROPRIATELY FOLLOWING THE CARDS STATEMENT. THIS DATA *
* IS IN THE FORM: volume of label / total cpm-1 / total cpm-2 / *
* nonspecific cpm-1 / nonspecific cpm-2 / mg tissue per *
* reaction cup (also could be mg protein or fraction of whole *
* tissue per cup) / age group or experiment number. *
*************** BY LANE J. WALLACE AS MODIFIED BY G. A. ORDWAY *
* *
* THE ABBREVIATIONS USED ARE:
* *
* VOL_LIG IS THE VOLUME OF RADIO-LIGAND ADDED TO ASSAY
* TOT_CPM IS THE TOTAL COUNTS
* NCPM IS THE NONSPECIFIC COUNTS
* VOL_SPK IS THE VOLUME OF THE SPIKE RADIO-LIGAND
* MG_TISS IS THE MILLIGRAM AMOUNT OF PROTEIN IN EACH ASSAY
* VOLASS IS THE VOLUME OF THE ASSAY IN LITERS
* TOT_MG IS THE TOTAL BINDING IN FMOLE PER MG PROTEIN
* NSP_MG IS THE NONSPECIFIC BINDING IN FMOLE PER MG PROTEIN
* CONC_LIG IS THE MOULAR CONCENTRATION OF FREE LIGAND
* FMOL_MG IS THE SPECIFIC BINDING IN FMOLE PER MG PROTEIN
* BD_FREE IS THE BOUND OVER FREE RATIO
* *
* ENTER DATA AS FOLLOWS:
* THE TWO SPIKE COUNTS SEPARATED BY A COMMA AND WITHIN THE *
* PARENTHESIS OF THE SPIKE = STATEMENT
* THE UL VOLUME OF THE RADIO-LIGAND ADDED TO THE SPIKE ASSAY IN THE *
* VOL_SPK = STATEMENT
* THE MG PROTEIN IN EACH ASSAY IN THE MG_TISS STATEMENT
* THE SPECIFIC ACTIVITY OF THE LIGAND IN THE ACTIVITY STATEMENT
* THE LITER VOLUME OF THE FINAL ASSAY MIX
* THE UL VOLUME OF THE RADIO-LIGAND ADDED TO ASSAY IN COLUMNS 1-4,
* THE TOTAL CPM IN COLUMNS 5-9 AND 10-14, AND THE NONSPECIFIC
* CPM IN COLUMNS 15-19 AND 20-25. THIS GOES IN THE LINES *
* FOLLOWING THE CARDS STATEMENT. THERE SHOULD BE ONE LINE OF *
* INPUT DATA FOR EACH DIFFERENT CONCENTRATION OF LIGAND.
* THE DATE OF ASSAY SHOULD BE UPDATED IN THE TITLE3 STATEMENT;

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OPTIONS LINESIZE=76;
DATA ONE;
  INPUT VOL_LIG 1-4 TCPM1 5-9 TCPM2 10-14 NCPM1 15-19 NCPM2 20-24
  MG_TISS 25-30 TIS $ 31-41;
  SPIKE = MEAN(18038,18644);
  VOL_SPK = 100;
  ACTIVITY = 30.2;
  VOLASS = 1.5E-3;
  MOLES = (1/0.4)*(1/2.2E12)*(1E-3/ACTIVITY);
  *TO CONVERT CPM TO MOLES:
  *CPM*1DPM/0.4CPM*1CI/2.2E12DPM*1E-3MOLE/{ACTIVITY}Cl;
  TCPM1 = TCPM1 - 24;
  TCPM2 = TCPM2 - 24;
  NCPM1 = NCPM1 - 24;
  NCPM2 = NCPM2 - 24;
  TOT_MG = SUM(TCPM1,TCPM2)/N(TCPM1,TCPM2)*MOLES/MG_TISS;
  NSF_MG = SUM(NCPM1,NCPM2)/N(NCPM1,NCPM2)*MOLES/MG_TISS;
  CONC_LIG=(VOL_LIG*SPIKE/VOL_SPK-SUM(TCPM1,TCPM2)/
            N(TCPM1,TCPM2))*MOLES/VOLASS/1E-9;
  FMOL_MG =(TOT_MG - NSF_MG)/1E-15;
  BD_FREE = FMOL_MG/CONC_LIG;
CARDS;
  5  152  144  26  24  2.96  YD-13B
  10  213  268  34  32  2.96  YD-13B
  20  396  386  45  52  2.96  YD-13B
  50  617  624  63  70  2.96  YD-13B
  100 908  713  83 104  2.96  YD-13B
  250 838  871 121 135  2.96  YD-13B
  5  220  214  25  30  3.92  MD-13B
  10  375  389  38  44  3.92  MD-13B
  20  587  619  56  50  3.92  MD-13B
  50  864  994  85  69  3.92  MD-13B
  100 1066 1096 120 113  3.92  MD-13B
  250 1293 1177 165 185  3.92  MD-13B
  5  293  268  27  32  5.01  OD-13B
  10  458  446  42  38  5.01  OD-13B
  20  754  747  47  41  5.01  OD-13B
  50 1191 1242  75  83  5.01  OD-13B
  100 1458 1445  85  69  5.01  OD-13B
  250 1555 1570 191 187  5.01  OD-13B
TITLE SCATCHARD ANALYSIS OF 3H-QNB IN BLADDERS OF Y, M, O RATS;
TITLE2 KD AND BMAX BASED ON MG TISSUE;
TITLE3 EXPERIMENT #13B;
TITLE4 BODY;
CAMS FILEDEF 20 DISK;
PROC PRINTTO UNIT=20;
  PROC GLM; BY TISNOTSORTED; MODEL BD_FREE=FMOL_MG;
PROC PRINTTO;
DATA REGI REGS;
INFILE FT20F001;
INPUT @2 NAME $ @;
PUT _INFILE_;
IF NAME='INTERCEP' THEN DO;
  INPUT INTCEP;
OUTPUT REGI;
END;
IF NAME='FMOL_MG' THEN DO;
INPUT SLOPE;
IF SLOPE=1 THEN DELETE;
DROP NAME;
OUTPUT REGS;
END;
DATA INTCEP;
SET REGI;
DROP SLOPE;
DATA SLOPE;
SET REGS;
DROP REGS;
DATA THREE; MERGE INTCEP SLOPE;
BMAX = (-INTCEP/SLOPE);
KD = -1/SLOPE;
PROC PRINT;
APPENDIX G

ANOVA SAS

************************************************************************************************************;
* *
* THIS PROGRAM PERFORMS BOTH A STUDENT-NEWMAN-KEULS TEST AND A *
* AND A DUNCAN'S MULTIPLE RANGE TEST AT VARIOUS LEVELS OF *
* PROBABILITY. DATA FILES CAN BE STORED SEPARATELY AND INSERTED *
* JUST BELOW THE CARDS STATEMENT. DATA FILES MUST BE FORMATTED *
* AS FOLLOWS: BEGINNING AT COLUMN 1, THE GROUP IDENTITY, USING *
* CHARACTERS AND NO SPACES; FOLLOWED BY A SINGLE SPACE AND THEN *
* A DATUM FOR THAT GROUP. EACH DATUM IS ON A SEPARATE LINE WITH *
* THE GROUP IDENTITY TYPED BEFORE IT. ANY NUMBER OF GROUPS CAN *
* BE USED.
* 
************************************************************************************************************;

OPTIONS LINESIZE=76;
TITLE DATA FROM DOXOTKMX DATA CONVERTED TO MM/GR TISSUE EMAX DATA;
DATA ONE;
INPUT AGE $ VARIABLE;
CARDS;
PROC SORT; BY AGE;
PROC MEANS N MEAN STDERR; BY AGE;
PROC GLM;
CLASS AGE;
MODEL VARIABLE = AGE;
MEANS AGE /SNK ALPHA=0.05;
MEANS AGE /SNK ALPHA=0.01;
MEANS AGE /SNK ALPHA=0.005;
MEANS AGE /SNK ALPHA=0.001;
MEANS AGE /DUNCAN ALPHA=0.05;
MEANS AGE /DUNCAN ALPHA=0.01;

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