EFFECT OF TEMPERATURE ON THE SENSITIVITY OF THE MOUSE VAS DEFERENS TO ADRENERGIC AGONISTS

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The isolated mouse vas deferens was used to evaluate possible mechanisms of supersensitivity to sympathomimetic amines produced by lowering the bath temperature. The effects of temperature were determined from cumulative concentration-response curves obtained from paired vasa deferentia at 37°C and 20°C. The effects of cocaine or tropolone pretreatment on the responses to (-)-norepinephrine were also examined.

Lowering the bath temperature to 20°C resulted in a shift of the norepinephrine concentration-response curve to the left and an increase in the maximum degree of contraction. Alteration of bath temperature did not affect the potency of methoxamine. However, the maximum degree of contraction to methoxamine was greater at 20°C than at 37°C. Cocaine pretreatment did not affect the potency of or the maximum degree of contraction to methoxamine at either temperature.

Pretreatment with pargyline or pretreatment with tropolone did not prevent the temperature-dependent supersensitivity to (-)-norepinephrine. Lowering the bath temperature increased the potency of (-)-phenylephrine and

(-)-alpha-methyl norepinephrine but did not significantly affect the maximum degree of contraction produced by either amine. Pretreatment with pargyline did not prevent the temperature-dependent sensitivity to (-)-phenylephrine. The potencies of (-)-alpha-methyl norepinephrine at 20°C and 37°C were not different after pretreatment with tropolone.

Pretreatment of the vas deferens with cocaine at 37°C resulted in a shift of the (-)-norepinephrine concentration-response curve to the left and an increase in the maximum response. Pretreatment with cocaine at 20°C did not alter the potency of (-)-norepinephrine and decreased the maximum degree of contraction to (-)-norepinephrine. After cocaine pretreatment, the -log molar ED_{50} and maximum contraction produced by (-)-norepinephrine at 20°C was not significantly different than the -log ED_{50} and maximum contraction at 37°C. Similar results were obtained when phenylephrine was used as the agonist.

Changing the temperature did not affect the values of the -log molar ED_{50} or the maximum contractions in response to (±)-alpha-methyl norepinephrine, (±)-metaraminol or (-)-metaraminol. Cocaine pretreatment also did not significantly alter the potency of (-)-metaraminol. Metaraminol and alpha-methyl norepinephrine were the least potent of the adrenergic agonists tested. Therefore, it is possible that the intraneuronal uptake of these compounds was saturated by concentrations approximating

those required to produce a response. In addition, these compounds have an indirect component of action. If intraneuronal uptake was decreased by cocaine or low bath temperature, indirect action would be markedly reduced. This would tend to obscure an effect of cocaine or low temperature on the ability of these amines to produce responses.

The retention of ^{14}C-norepinephrine by the mouse vas deferens was lower at 20°C than at 37°C. Pargyline or tropolone pretreatment did not affect the retention of ^{14}C-norepinephrine. After cocaine pretreatment, the tissue/medium ratios at 20°C and 37°C were equivalent.

In conclusion, the increase in the maximum response of the mouse vas deferens to adrenergic agonists as a result of cocaine pretreatment appears to be related to the ability of cocaine to inhibit intraneuronal uptake of the adrenergic agonists. It is suggested that this increase in the maximum response after cocaine pretreatment may be a result of activation of a greater number of receptors by the adrenergic agonist. In addition, the data suggest that reducing the bath temperature from 37°C to 20°C increases the sensitivity of the vas deferens to adrenergic agonists by a mechanism similar to that of cocaine pretreatment. The exception to this conclusion is the increase in the maximum response to methoxamine at 20°C. Several possible explanations for the increased maximum degree of contraction to methoxamine are proposed.

APPROVED Carl L. Buckner

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INTRODUCTION

Isolated smooth muscle preparations are often utilized to study both the autonomic nervous system and the mechanisms involved in smooth muscle contraction. There are several reasons for using *in vitro* rather than *in vivo* smooth muscle preparations for these studies. The *in vitro* preparation eliminates the variability of drug distribution and disposition encountered *in vivo*, thereby making it easier to determine the concentration of drug in contact with the tissue. In addition, it eliminates possible alterations of the organ's response caused by interaction of the drug with sites outside of the organ, or by the influence of neural or hormonal feedback systems. Finally, in an *in vitro* preparation, it is often easier to measure the actual tissue response (Furchgott, 1968).

The response of an isolated smooth muscle to an agonist is thought to be a consequence of an agonist-receptor interaction. Although the actual concentration of drug in the region of the receptor is not known, it is assumed to be proportional to the concentration of drug in the bath fluid (Ariens *et al.*, 1964). The relationship of the drug concentration to the muscle response is usually represented graphically by plotting the log of the drug concentration in the bath on the abscissa and the response on the ordinate. The resultant concentration-response relationship is a sigmoid curve which is nearly linear in

the region of half-maximal response (ED_{50}). The affinity of the drug for the receptor is proportional to the -log ED_{50} and the intrinsic activity of the drug is proportional to the maximal response obtained with the drug (Ariens et al., 1964).

In any given tissue, true receptor affinity and intrinsic activity of an agonist are relatively constant. However, apparent receptor affinity or intrinsic activity of an agonist can be altered in a number of ways. In general, there are three ways to alter the apparent affinity of an adrenergic agonist for its receptor. The first, which will be discussed more fully in another section, is to alter the mechanisms responsible for termination of the activity of the agonist, thereby increasing or decreasing the proportion of agonist that reaches the receptor area. The second is to alter the receptor itself and thereby increase or decrease the affinity of the agonist for the receptor. The third is to alter the tissue at some point other than the receptor, thereby changing the ability of the muscle to contract in response to a given quantum of receptor excitation.

The intrinsic activity of an agonist, as estimated by the magnitude of the maximum response, is not usually considered to be affected by the concentration of the agonist at the receptor. Any increase in the maximum response to an agonist is believed to be due to a change in the receptor itself, an increase in the total number of

receptors available, or an alteration in this tissue at some site other than the receptor. However, the nature of the site referred to as a receptor and the series of reactions that link the agonist-receptor interaction to the response are not known.

The sequence of events that follows the reaction of an excitatory drug with a smooth muscle receptor has been reviewed by Hurwitz and Suria (1971). At this time it is not possible to give one simple model for the steps involved in the contraction of smooth muscle since a variety of effects may be observed, depending upon the agonist and smooth muscle employed. For example, in some muscles the complexation of an excitatory agonist with a receptor results in a decrease in the magnitude of the membrane potential. However, excitatory agents are often capable of producing a contraction even after the muscle has been depolarized with K_2SO_4 (Evans and Schild, 1957). In both cases though, it is believed that the agonist in some way induces a membrane change that permits the entrance of calcium ions into the cytoplasm (Hurwitz and Suria, 1971).

There are probably two main sources of calcium ion that are important in smooth muscle contraction, a tightly bound, intracellular pool and an extracellular pool. The relative importance of each varies with the agonist and smooth muscle employed (Hurwitz and Suria, 1971; Hurwitz et al., 1973). The contractile response of the rabbit

aorta to norepinephrine requires intracellular calcium, while the potassium-induced contraction requires extracellular calcium (Devine et al., 1972). In contrast, the contractile responses of the rat vas deferens to norepinephrine and potassium are both dependent upon the extracellular calcium ion (Chang et al., 1971; Janis and Triggle, 1971). Further evidence of the importance of calcium in the contractile responses of the vas deferens comes from the studies by Bennett (1967) of the effects of sodium and calcium on the action potential evoked by intracellular electrical stimulation and by hypogastric nerve stimulation. These studies suggested that calcium ions may carry the current to generate the action potential in this tissue. Therefore, it is possible that an increase in the maximum response of a tissue such as the vas deferens could be due to an alteration in the availability of calcium ion. This may be a result of either receptor changes or changes in the permeability of the muscle membrane. Alteration in the availability of Ca^{++} is, however, only one example of how the factors involved in the contractile process may affect the maximum degree of contraction.

The contractile mechanism as a whole is not well understood and a number of explanations for changes in the maximum response could be proposed. For example, another possible explanation for the increased height of contraction is decreased activity of the processes

responsible for the termination of the contractile response. A prolongation of the contractile response coupled with an increase in the number of agonist-receptor complexes could result in an increase in the maximum contraction. Because of the uncertainty of the mechanism involved in muscle contraction, a direct method for testing these hypotheses is not available. Therefore, the response can only be characterized by indirect measurements.

Mechanisms responsible for the termination of the action of catecholamines

Mechanisms responsible for the termination of the activity of sympathomimetic amines influence the concentration of adrenergic agonist at the receptor. Mechanisms of catecholamine inactivation identified in isolated tissues include metabolism by the enzymes monoamine oxidase (MAO) and/or catechol-O-methyl transferase (COMT), and physical removal from the receptor area by means of intraneuronal or extraneuronal uptake. These prejunctional determinants of the potency of an agonist can be studied more readily than post-junctional mechanisms, and many of their properties have been defined (Trendelenburg, 1972c).

The characterization of the enzyme MAO has been hindered by its localization as an insoluble component of the outer membrane of the mitochondrion (Schnaitman

et al., 1967). Within recent years, the enzyme has been isolated from liver and brain tissue and partially characterized. It is generally recognized as containing one mole of the flavin cofactor, FAD, per 120,000 g of protein (Sandler and Youdim, 1972). A metal requirement has not been proven, but nutritional iron does appear to be necessary for the activity of rat liver MAO (Symes et al., 1971). The characterization has been further complicated by the growing evidence that multiple forms of MAO exist. Several forms of MAO which vary in their specificity for substrate and for inhibitors may be present in a single tissue type. The evidence for the existence of multiple forms of the enzyme as well as a discussion of their functional significance has been reviewed recently (Sandler and Youdim, 1972).

The metabolism of norepinephrine by MAO results in the loss of the amine group and formation of 3,4-dihydroxymandelic aldehyde, which can undergo further oxidation or reduction and/or be further metabolized by COMT. Phenethylamines with a methyl group on the alpha carbon are not substrates for MAO. The activity of MAO can be inhibited by compounds such as tranylcypromine, iproniazid, pargyline and harmine.

Monoamine oxidase is located in postganglionic sympathetic neurons as well as extraneuronally. Jarrott and Iversen (1971) noted that after denervation, a procedure which results in complete degeneration of

the adrenergic nerve endings, the vasa deferentia of the rat, guinea-pig, and rabbit retained approximately fifty per cent of the activity of MAO. In contrast, there was no measurable activity of DOPA decarboxylase and tyrosine hydroxylase, enzymes necessary for synthesis of norepinephrine. These results indicated that DOPA decarboxylase and tyrosine hydroxylase are located intraneuronally, while MAO is located both intraneuronally and extraneuronally.

Cathechol-O-methyl transferase, another enzyme involved in metabolism of norepinephrine, has been partially purified from the rat liver. Its activity is dependent upon the presence of a divalent ion, preferably Mg^{++}, and S-adenosylmethionine, a methyl donor. Catechols and polyphenols are substrates for the enzyme, but monophenols are not (Axelrod and Tomchick, 1958). Inhibitors of COMT include polyphenols (Axelrod and LaRoche, 1959) and tropolone (Belleau and Burba, 1963). As with MAO, COMT has multiple forms. At least two separate forms of the enzyme have been identified (Axelrod and Vesell, 1970).

In contrast to MAO, which is found in mitochondria, the highest concentrations of COMT are found in the soluble fraction of the cell. Most COMT activity is not associated with the neuron but is located extraneuronally (Carlsson and Hillarp, 1962). However, evidence that small amounts of COMT are located intraneuronally has

been reported by Jarrott (1971). He noted a decrease in the activity of COMT after sympathetic denervation of the rat and rabbit vasa deferentia, the cat nictitating membrane, and the rabbit submaxillary gland. However, there was no change in the activity of COMT after denervation of the mouse heart or spleen or of the guinea-pig vas deferens. At high concentrations of S-adenosylmethionine, K_m values for COMT from the normal and denervated vasa deferentia were different, a finding which suggests that neuronal and extraneuronal COMT in this organ have different kinetic properties. Since the concentration of COMT located intraneuronally may be considerably lower than that located extraneuronally, it will be difficult to determine which nerves contain COMT until a method is devised to visualize it in the tissues. The functional significance of variations in the localization or form of COMT is not known. These factors should be kept in mind, however, when evaluating physiological data.

The most important means of terminating the action of catecholamines is uptake by the adrenergic neuronal membrane (Trendelenburg, 1972c). There are two stages of intraneuronal uptake. The first stage is uptake of norepinephrine through the membrane of the neuron into the cytoplasm, where norepinephrine can either be metabolized by MAO or undergo the second stage of uptake into the intraneuronal vesicles. The structural

requirements and stereoselectivity of membranal uptake differ from those of vesicular uptake (Von Euler and Lishako, 1967). For example, vesicular uptake is always stereospecific (Von Euler and Lishako, 1967), but the degree of stereoselectivity of membranal uptake seems to be tissue and species dependent. By measuring arterio-venous differences in the rabbit heart, Draskóczy and Trendelenburg (1968) demonstrated that uptake through the neuronal membrane of this organ was not stereospecific. With a similar method, Jarrott and Iversen (from Trendelenburg, 1972b) showed that uptake through the neuronal membrane was stereoselective in the rat heart but not in the guinea-pig heart. Therefore, it appears that it is not possible to predict the degree of stereoselectivity of membranal uptake for a particular tissue.

The uptake of norepinephrine into the neuron can be inhibited by a variety of compounds such as phenoxybenzamine, desipramine and cocaine. In addition, a number of sympathomimetic amines can compete with norepinephrine for the neuronal membrane uptake site. Some, but not all, of these sympathomimetic amines can be accumulated by the neuron (Iversen, 1967). The structure-activity relationships of the phenylethylamines which inhibit the neuronal uptake of norepinephrine in the rat heart have been summarized by Iversen (1967).

In general, the affinity of a compound for the uptake process is enhanced by the substitution of methyl groups on the alpha carbon or by phenolic hydroxyl groups in the para- and meta-positions. Beta-hydroxylation, N-substitution, or O-methylation of phenolic hydroxyl groups decreases the affinity for the neuronal uptake site. From these structure-activity studies it is apparent that (-)-metaraminol and (-)-alpha methyl norepinephrine can inhibit the uptake of norepinephrine. In contrast, (±)-isoproterenol, (±)-normetanephrine and (±)-methoxamine have a low affinity for the neuronal uptake process.

The fourth major means of terminating the activity of norepinephrine in the region of the adrenergic receptor is extraneuronal uptake. This mechanism of inactivation was first described by Iversen (1965) as functioning only in the presence of high concentrations of exogenous catecholamines. After further study, the description of the process was revised (Lightman and Iversen, 1969). Extraneuronal accumulation of catecholamines begins at low concentrations but this is not evident unless COMT and MAO are inhibited. It is particularly pronounced at high concentrations of catecholamines. The main cell types which retain norepinephrine are collagen and smooth muscle cells (Gillespie et al., 1970). However, there exist considerable species and organ differences in the ability

of these tissues to retain norepinephrine extraneuronally (Gillespie and Muir, 1970; Burnstock et al., 1972). For example, the non-arterial smooth muscle of the mouse and rabbit vasa deferentia can retain high levels of norepinephrine, while the guinea-pig and rat vasa deferentia retain very little. The reasons for these differences are still under study.

The structural requirements for extraneuronal uptake in the rat heart are quite different from those for intraneuronal uptake (Iversen, 1967). Normetanephrine and metanephrine, phenylethylamines which have a low affinity for intraneuronal uptake, have a high affinity for the extraneuronal uptake process and can inhibit the extraneuronal uptake of norepinephrine. In addition, a number of compounds unrelated to phenylethylamines can inhibit extraneuronal uptake of norepinephrine. Examples of these inhibitors of extraneuronal uptake are phenoxybenzamine (Iversen, 1967) and corticosteroids such as corticosterone, 17-beta-estradiol and testosterone (Iversen and Salt, 1970).

Of the mechanisms for termination of the activity of norepinephrine, the most important, in most tissues, appears to be intraneuronal uptake (Trendelenburg, 1972c). An exception to this rule would be tissues such as the rabbit aortic strip, where the density of adrenergic innervation is very low. In this tissue, extraneuronal uptake with subsequent metabolism by MAO and COMT,

appears to be most important (Kalsner and Nickerson, 1969a; Kalsner and Nickerson, 1969b). Despite the importance of uptake, it should be remembered that at all times there is a functional equilibrium among the several means of inactivation. In some tissues inhibition of COMT has no effect upon the sensitivity of the tissue to adrenergic agonists. However, when neuronal uptake and MAO are inhibited, changes in the activity of COMT affect the sensitivity of the tissue to the adrenergic agonist. In addition, in some tissues the quantity of O-methylated metabolites formed as a result of neural stimulation increases when MAO is inhibited (Tarlov and Langer, 1971; Trendelenburg et al., 1971). Another consideration is that the apparent activity of an enzyme may be altered by inhibition of one of the uptake mechanisms, resulting in decreased access of norepinephrine to sites of enzymatic activity. Conversely, inhibition of MAO may reduce the uptake of norepinephrine. Since inhibition of MAO decreases the intraneuronal metabolism of norepinephrine, norepinephrine accumulates in the cytoplasm of the nerve. Eventually the intraneuronal concentration of norepinephrine is high enough so that additional norepinephrine cannot be accumulated (Trendelenburg and Draskóczy, 1970; Graefe et al., 1971). A consideration of these interrelationships should make it clear that if inhibition of any one process of inactivation does not lead to increased sensitivity of a

tissue, it does not prove that this process is unimportant in terminating the activity of the adrenergic agonist in that tissue.

Changes in tissue sensitivity as a result of denervation, decentralization, or cocaine

One method for determining the function of peripheral autonomic nerves is to cut the nerves, let them degenerate, and then correlate the changes in response with the microscopically or biochemically determined alterations of the tissue. In studies of this type, severing the postganglionic neuron is referred to as denervation and severing the preganglionic neuron is called decentralization. While conducting studies of this type several investigators have noted that severing the nerves to some tissues has resulted in increased sensitivity of the tissue to exogenous compounds. For example, denervation of the cat nictitating membrane leads to a supersensitivity consisting of two components. The first component, which resembles the effects of pretreatment with cocaine, develops rapidly as the adrenergic nerve fibers degenerate. The second component is similar to decentralization supersensitivity and continues to increase up to four weeks after surgery (Trendelenburg, 1963; Trendelenburg, 1966; Langer et al., 1967). The cocaine-like component is specific for amines which are taken up by nerve

endings; in contrast, the decentralization component results in potentiation of not only catecholamines but of compounds such as acetylcholine (Trendelenburg and Weiner, 1962), serotonin (Pluchino, 1972), and methoxamine (Trendelenburg et al., 1970). Therefore, the cocaine-like component of denervation supersensitivity is considered to be due to decreased neuronal uptake, while the decentralization component is considered to be due to a postjunctional alteration of the tissue (Trendelenburg, 1972c).

The reason for the postjunctional change after decentralization has not been determined. Green and Fleming (1967) demonstrated that the postjunctional effects were not due to changes in the alpha receptor since the antagonism of norepinephrine by phentolamine was not altered by denervation, decentralization or cocaine pretreatment. Therefore, the increased sensitivity may be due to an alteration in the total number of receptors or to an alteration in the excitation-contraction coupling mechanisms.

Increased sensitivity after denervation, decentralization and cocaine pretreatment has also been observed in other tissues such as the vas deferens. The results, however, have not been as conclusive as with the cat nictitating membrane. After denervation, the concentration-response curve of the vas deferens to norepinephrine is shifted to the left. There appear to

be two components in this shift of the norepinephrine concentration-response curve. One component of the shift is specific for catecholamines while the second affects both catecholamines and other agonists such as acetylcholine, oxymetazoline, histamine and K^+ (rat, Birmingham et al., 1970; rat, Kasuya et al., 1969; guinea-pig, Westfall et al., 1972). The non-specificity of this second component suggests that it is due to a postjunctional change. Further evidence for this has been obtained by Westfall et al. (1972) by observing the effects of decentralization on the concentration-response relationships of the guinea-pig vas deferens to norepinephrine and histamine. The concentration-response curve of both of these compounds is shifted to the left, but there is no increase in the maximum response. The similarity of the non-specific shifts observed after denervation and decentralization suggests that they may be a result of a similar postjunctional alteration.

In addition to a shift to the left of the concentration-response curve, denervation of the vas deferens also results in an increase in the maximum response to norepinephrine, acetylcholine, and histamine but not to K^+ (guinea-pig, deMoraes et al., 1972; Westfall et al., 1972; rat, Kasuya et al., 1969). The reason for the increase in the maximum response has not been determined. However, Krell and Patil (1972) obtained similar potency ratios for the isomers of

norepinephrine, suggesting that denervation of the rat vas deferens did not cause a qualitative change in alpha adrenergic receptors. Therefore, postjunctional changes may occur in the sequence of events subsequent to receptor activation. Westfall et al. (1972) suggested that the increase in maximum response may be related to the decrease in the number of nerve endings. It was proposed that this may increase the number of nexal regions, the areas along adjacent smooth muscle cells where the cell membranes are in very close apposition, thereby improving the cell-to-cell communication. Another proposed reason for the increased maximum response is that denervation increases Ca^{++} mobilization (Kasuya et al., 1969). With present techniques both of these hypotheses are difficult to study.

In some tissues, pretreatment with cocaine also has been shown to cause an increase in the maximum response. The shift to the left of the concentration-response curve to norepinephrine after cocaine pretreatment is generally believed to be due to the ability of cocaine to inhibit the uptake of norepinephrine through the neuronal membrane. The possibility of a postjunctional effect of cocaine has been discussed extensively. The evidence for and against a postjunctional effect has been reviewed by Trendelenburg (1972a) and Maxwell and Eckhart (1972). Briefly, it does not appear that cocaine has a postjunctional effect on the cat nictitating

membrane (Trendelenburg, 1970). However, in the rabbit aorta, Maxwell et al. (1966) reported that the inhibition of uptake of norepinephrine by cocaine does not correlate completely with the increase in maximum response. In addition, Kalsner and Nickerson (1969c) reported that cocaine increased the maximum response of the rabbit aorta to methoxamine. Since methoxamine has a very low affinity for neuronal uptake, inhibition of neuronal uptake by cocaine should not affect the concentration of methoxamine at the receptors. Therefore, the increased maximum contraction would have to be a result of some other mechanism. The data suggest that there is a tissue variation and that cocaine may indeed have a post-junctional effect in the rabbit aorta.

The studies of the effect of cocaine on the vas deferens have led to a variety of results and conclusions. Westfall et al. (1972) and Wakade and Krusz (1972) observed no increase in the maximum response of the guinea-pig vas deferens to norepinephrine after cocaine pretreatment. However, with the rat vas deferens, Barnett et al. (1968), Buckner et al. (1969), Varma and McCullough (1969), Kasuya and Goto (1968), Greenberg and Long (1971) and with the guinea-pig vas deferens, deMoraes et al. (1970), observed an increase in the maximum response to norepinephrine. Cocaine pretreatment also increased the maximum responses of the rat vas deferens to Ba^{++}, K^{+}, acetylcholine, and Ca^{++} (Greenberg

and Long, 1971). Reserpine pretreatment blocked the increases in the maximum response to K^+, Ba^{++}, and acetylcholine but not the responses to Ca^{++} or norepinephrine. This supports the hypothesis that cocaine may have an effect on the amount of Ca^{++} available for contraction.

Changes in sensitivity of isolated tissues due to changes in temperature

Changes in temperature can affect a number of factors involved in the response of adrenergically innervated tissues. Lowering the bath temperature decreases the activity of MAO and COMT and decreases the capacity for intraneuronal and extraneuronal uptake (Fuhrman et al., 1944; Oppermann et al., 1972; Iversen, 1967; Gillespie et al., 1970). In addition, temperature changes may affect the postsynaptic factors involved in the response of a tissue. Experiments by Kunos and Szentivanyi (1968) and Buckley and Jordan (1970) suggest that decreasing the bath temperature results in a transformation of beta-receptors to alpha-receptors in the frog and rat heart. Changes in temperature also may alter the ionic content of tissues (Freeman-Narrod and Goodford, 1962).

A number of investigators have studied the effects of temperature on the K^+ and Na^+ content of tissues. Decreasing the bath temperature increases the level of Na^+ and decreases the level of K^+ in tissues (guinea-pig

taenia-coli, Freeman-Narrod and Goodford, 1962; rat heart, Mendler et al., 1971; dog heart, Bui-Mong-Hung et al., 1972). Reisin and Gulati (1972) showed that in the guinea-pig taenia-coli there is a small but gradual decrease in the level of intracellular K^+ as the temperature is lowered from 37°C to 17.5°C. However, when the temperature is lowered from 17.5°C to 12.5°C a rapid exchange of K^+ and Na^+ occurs. At 13.8°C the level of K^+ in the tissue had decreased to one-half of its value at 36°C. These changes in Na^+ and K^+ are probably a result of a decrease in the activity of the Na-K-ATPase and therefore a decrease in the transport of Na^+ out of the tissue. These ionic alterations in tissues result in a decrease in the membrane potential and may affect the functional response of the tissue.

Temperature also has an effect on the level of Ca^{++} in tissues. Several investigators have reported that lowering the temperature increases the availability of Ca^{++} for contraction (guinea-pig atria, Lahrtz et al., 1967; guinea-pig and cat atria, Sumbera et al., 1967; turtle oviduct, Somlyo et al., 1971). Lahrtz et al. measured the turnover of ^{45}Ca and total Ca^{++} content of the guinea-pig atria at 30°C, 15°C, and 1°C. They noted that as the temperature decreased the total Ca^{++} content of the atria increased. At 15°C the release of ^{45}Ca from the tissue was inhibited but ^{45}Ca uptake was only slightly diminished. In addition, Devine et al. (1972)

reported that the ability of the main pulmonary artery and mesenteric vein of the rabbit to contract at 23.5°C in Ca^{++} free solution was directly related to the volume of sarcoplasmic reticulum in these tissues. The sarcoplasmic reticulum of these tissues is thought to be a storage area for intracellular Ca^{++} (Hasselbach, 1966). In contrast, Tomita (1970) reported that the guinea-pig taenia-coli apparently lost Ca^{++} from the membrane at low temperatures.

In physiological studies of isolated mammalian tissues, both supersensitivity and subsensitivity to catecholamines have been reported at low temperatures. The observed differences in the effect of temperature on different tissues suggest that there is probably a number of mechanisms responsible for such changes in sensitivity. Temperature alteration may produce several changes in tissues. The importance of these changes in affecting the response of the tissue may depend upon the tissue, the species and the type of stimulus used.

Most studies on the effects of temperature on isolated tissues have been conducted on tissues of the gastrointestinal tract or the cardiovascular system. Lowering the bath temperature reduces the number of spontaneous contractions of the longitudinal muscle of the rabbit duodenum and ileum (Small and Weston, 1971; Wagner et al., 1972) and the guinea-pig taenia-coli (Bulbring and Kuriyama, 1963). Wagner et al. (1972)

noted that, although the number of spontaneous contractions of the rabbit ileum was less at 25°C than at 37°C, the maximum tension developed was the same at both temperatures. In addition, the ileum was more sensitive to the relaxing effects of isoproterenol at 25°C than at 37°C. In contrast, Devine et al. (1972) reported that maximal tension developed by the rabbit taenia-coli in response to acetylcholine was less at 23.5°C than at 37°C.

Various tissues of the cardiovascular system also respond differently to a change in temperature. Devine et al. (1972) reported that the maximal tension developed by the main pulmonary artery, thoracic aorta and mesenteric vein of the rabbit in response to norepinephrine was less at 23.5°C than at 37°C. In contrast, lowering the bath or perfusion temperature resulted in a constriction of the hepatic artery and portal vein of the isolated dog liver (Absalon et al., 1971) and increased constriction of the rabbit ear artery in response to norepinephrine or electrical stimulation (Martin and Wallace, 1971). In addition, Vanhoutte and Shephard (1970b) reported that decreasing the bath temperature to 29°C resulted in augmentation of venoconstrictor and venodilator actions of isoproterenol and norepinephrine on the saphenous vein of the dog. Besides augmenting the degree of constriction of the isolated cutaneous veins of the dog in response to

catecholamines, decreasing the temperature from 43°C to 25°C also augmented the contractile responses to acetylcholine, serotonin and electrical stimulation (Vanhoutte and Shephard, 1970a). Heyndrickx and Vanhoutte (1971) determined that this potentiation of the response to electrical stimulation was independent of the external Ca^{++} concentration.

Studies on chronotropic responses of mammalian atria have emphasized the species differences in response to alteration in temperature. The isolated rabbit atria is less sensitive to catecholamines at 26°C than at 37°C; however, the response of the rat atria to norepinephrine is unaffected by the same temperature changes (Oppermann, 1970). In contrast, the sensitivity of the guinea-pig or mouse atria to catecholamines is greater at 26°C or 27°C than at 37°C (Trendelenburg, 1968; Oppermann et al., 1972; Muñoz-Ramirez et al., 1973). This increased sensitivity of the guinea-pig and mouse atria to catecholamines appears to be a result of a decrease in the activity of COMT at 26°C (Oppermann et al., 1972; Muñoz-Ramirez et al., 1973).

Since the vas deferens has a very dense adrenergic innervation (Sjostrand, 1965), this tissue is often used to study the effect of drugs or various pretreatments on the adrenergic system. Experiments involving temperature changes have not been designed to determine the effects of temperature on the vas deferens but have utilized

temperature variations to characterize the nature of the response to stimulation. Low frequency preganglionic hypogastric nerve stimulation and transmural stimulation of the guinea-pig vas deferens initiate a response which can be potentiated by reducing the bath temperature (Della Bella et al., 1965; Ambache and Aboo Zar, 1971; Swedin, 1971; Birmingham and Freeman, 1972). The first (rapid) response component is potentiated to a greater extent than the second (slow) component. This suggests that the two components may be a result of activation of different types of nerve fiber (Birmingham and Freeman, 1972; Ambache and Aboo Zar, 1971). Ambache and Aboo Zar (1971) noted that reducing the temperature from 35°C to 20°C resulted in potentiation of the effects of muscarine on the guinea-pig vas deferens. The response to muscarine was not potentiated as much as the contraction due to electrical stimulation; however, it is not clear whether the response to more than a single concentration of muscarine was examined. The potentiation of the muscarine contractions suggested that temperature may have an effect on the smooth muscle itself as well as on neurotransmission.

In general, studies on the effects of temperature on responsiveness of the vas deferens have not made use of the complete concentration-response curve produced by adrenergic agonists. However, one study by Krell and Patil (1972) suggested that reducing the temperature to

30°C tended to decrease the sensitivity of the rat vas deferens to norepinephrine. An investigation of the responses of paired vasa deferentia over a wide temperature range is needed for characterization of the effects of temperature on this adrenergically innervated tissue.

The innervation of the vas deferens

Studies of the physiology of the vas deferens have been conducted since 1858 (from Sjostrand, 1965). The current increased interest in this muscle originated from the development of the guinea-pig vas deferens-isolated hypogastric nerve preparation of Hukovic (1961). Studies of this preparation stimulated a variety of investigations of the innervation of the vas deferens and the use of this tissue as a tool to study adrenergic mechanisms. There are several advantages inherent in using the vas deferens to study the adrenergic nervous system, such as, the high density of adrenergic innervation, the ability to stimulate both preganglionic and postganglionic neurons in an isolated preparation, and the ease of measuring the response.

The vasa deferentia of the mouse, rat and guinea-pig are innervated through the hypogastric nerve. In contrast to the adrenergic innervation of most tissue, the sympathetic ganglia are located in close proximity to the muscle; therefore, the postganglionic neurons are

unusually short. In addition, these short postganglionic nerves have an unusually dense concentration of varicosities which results in a high concentration of norepinephrine in the tissue (Birmingham and Wilson, 1963; Sjostrand, 1965). The terminal axons of the post-ganglionic adrenergic fibers penetrate the bundle of smooth muscle cells of the vas deferens. This is in contrast to the terminal axons of other smooth muscles such as the intestine where the axons are confined to the extracellular space surrounding the bundle of smooth muscle cells (Holman, 1970). The majority of the adrenergic receptors of the vas deferens appear to be of the alpha type. However, there is a minor beta adrenergic component which is inhibitory (Large, 1965; Takagi and Takayanagi, 1965; Ganguly and Bhattacharya, 1969).

In the last few years, increasing evidence for a significant cholinergic innervation has been presented. Clementi et al. (1969) reported that neostigmine, a competitive acetylcholinesterase (AChE) inhibitor, potentiated the response to hypogastric nerve stimulation and that this response was blocked by atropine. In addition, Robinson (1969) determined that about twenty-five per cent of the fibers innervating the vas deferens contained AChE. More recently, Knoll et al. (1972) showed that acetylcholine was released during field stimulation of the rat vas deferens. However,

cholinergic innervation is thought to be a minor component in neural transmission to the vas deferens.

Though it is convenient to discuss the studies of the guinea-pig, rat and mouse vasa deferentia together, it should be noted that significant anatomical and electrophysiological differences between these tissues exist. For example, in the rat and mouse, the spike potential is graded and not actively propagated through the tissue. However, in the guinea-pig vas deferens, the spike potential is all-or-none and is propagated through the tissue (Furness and Burnstock, 1969). In addition, the refractory period is longer for the guinea-pig vas deferens than for the mouse and rat. Furness and Burnstock (1969) suggested that the difference in spike activity may be correlated with the anatomical differences. According to Merrillees (1968) only one-half of the muscle cells of the guinea-pig vas deferens are directly innervated by axons whose terminals are located within 200 Å of these cells. This is in direct contrast to the vasa deferentia of the rat and mouse where every smooth muscle cell is directly innervated by at least one axon (Richardson, 1962; Yamauchi and Burnstock, 1969). Therefore, in the guinea-pig vas deferens the excitatory junction potential from an innervated smooth muscle cell is "electrotonically coupled" to neighboring cells, but in the rat and mouse vas deferens where each muscle is innervated separately,

the spike potentials are initiated in individual cells (Furness and Burnstock, 1969).

In summary, interest in the responses of the vas deferens to electrical stimulation and various agonists has increased during the past few years due to the high concentration of adrenergic innervation of this organ. However, the isolated vas deferens, which in 1961 appeared to be a completely sympathetically innervated organ, has proved to be much more complex than originally believed. A number of studies have revealed that, besides a large number of alpha adrenergic receptors, the vas deferens also contains a few beta adrenergic, as well as, cholinergic receptors. In addition, the unique relationship between the nerves and the smooth muscle cells of the vasa deferentia of the mouse and guinea-pig has been correlated with their responses to electrical stimulation. Despite its unique properties, studies of the vas deferens and its innervation will hopefully provide more information about the nervous system in general.

Statement of the problem

Recently there has been increased interest in the effects of temperature on the physiological responses of tissues to drugs or electrical stimulation. Much of this interest has resulted from the use of hypothermia as an adjunct to open heart surgery. However, reduced

temperature is also used as a method for preserving tissues and as an experimental tool to separate different mechanisms involved in physiological or pharmacological responses.

A few in depth studies on the effect of temperature on the responses of isolated tissues to adrenergic amines have been conducted. Most of these studies have examined the effects of temperature on responses mediated via activation of beta adrenergic receptors. While some investigators attribute the increased sensitivity at low temperatures to alteration of beta receptor conformations, others believe the supersensitivity to be due to inhibition of a saturable process responsible for removal of the adrenergic agonist from receptor regions (see the Introduction). Fewer studies have been reported regarding the influence of temperature on smooth muscle contractile responses elicited through alpha adrenergic receptors. Investigation of these systems might be expected to further elucidate the mechanisms of temperature-dependent sensitivity to catecholamines.

For several reasons, the isolated mouse vas deferens was selected as the test system for analysis of effects of temperature on action of adrenergic amines. First, the vas deferens contains primarily alpha adrenergic receptors. The mechanisms involved in responses to alpha receptor activation have not been as well studied as the mechanisms mediating responses to beta receptor

activation. For most beta receptor mediated responses, cyclic-3',5'-AMP appears to be an intermediate. In contrast, the level of cyclic-3',5'-AMP does not appear to be increased by alpha receptor activation. If decreasing the bath temperature, increases the sensitivity of tissues to beta receptor agonists by decreasing the metabolism of cyclic AMP, temperature alterations may not be expected to markedly influence responses mediated by alpha receptor activation. Second, in the vas deferens, alpha receptor activation produces a contractile response. Most of the in depth studies of the effect of temperature on the responses of isolated smooth muscles to adrenergic agonists have previously been concerned with inhibitory processes. Therefore, it is of interest to study the effects of temperature on a contractile response. Third, the role of Ca^{++} in the chronotropic response of isolated atria to adrenergic agonists has not been well defined. However, in the vas deferens, the role of Ca^{++} in the responsiveness of the muscle has been studied extensively. Since the role of Ca^{++} in the response of the vas deferens is more clearly defined, a study of the effect of temperature on this tissue may help to clarify the role of Ca^{++} in temperature-dependent sensitivity changes. Fourth, the vas deferens has a very dense adrenergic innervation. This implies that norepinephrine has an important role in the physiological response of this tissue. In addition,

the density of adrenergic innervation suggests the presence of active mechanisms for terminating the actions of endogenous neurotransmitter. For these reasons, it was decided to study temperature-dependent supersensitivity of the mouse vas deferens utilizing a number of adrenergic agonists and various pretreatments.

MATERIALS AND METHODS

Tissue preparation

Albino Swiss-Webster mice (C. L. Rolfsmeyer Co., Madison, Wisconsin) weighing 35 to 45 gms were killed by cervical dislocation. The vasa deferentia were removed and placed in a 100 ml water-jacketed dissection bath. The bath contained a physiological salt solution of the following composition (mM): $NaHCO_3$, 21.9; KH_2PO_4, 1.2; NaCl, 116.5; $MgCl_2 \cdot 6\ H_2O$, 1.2; $CaCl_2 \cdot 2\ H_2O$, 2.5; KCl, 3.7; Dextrose, 49.2 (Vohra, 1970) dissolved in deionized distilled water. The solution was aerated with 95% O_2 and 5% CO_2 through a sintered glass filter. The temperature in the dissection bath was maintained by circulating distilled water from a five gallon reservoir through the outer jacket of the bath. The reservoir water was kept at a temperature of $37 \pm 1^{\circ}C$ or $20 \pm 1^{\circ}C$ with a Tecam heater pump and a copper coil through which cold tap water was circulated.

The tissue was dissected on a ground glass plate and was frequently immersed in the physiological solution. The mesenteric coat was stripped from the vas deferens and a 1.5 cm segment was removed from the epididymal end of the muscle. Since occluding the ends of the vas deferens increased the variability of the data, the ends of the segment were cut diagonally and threads tied to each tip.

The vas deferens was suspended in a 10 ml water-jacketed tissue bath by tying one end to a glass support rod and the other to a lightweight isotonic lever (20:1 amplification). The lever, which was mounted on a concentric vernier tension adjuster, was regulated to apply 150 mg of tension to the tissue. Lever deflections resulting from drug-induced contractions were recorded on a smoked drum kymograph.

Construction of log concentration-response curves

The paired vasa deferentia were allowed to equilibrate in the physiological salt solution for twenty minutes prior to obtaining the cumulative concentration-response curves to adrenergic agonists. The solutions of agonists used for construction of the concentration-response curves were 1:10 dilutions of a 7.2×10^{-2} M solution. The volume of each solution added to the bath and the final bath concentrations achieved are presented in Table 1. The concentration in the bath was increased only after the response to each concentration of agonist had reached its maximum. The vas deferens was considered to have reached its maximum degree of contraction to an agonist when further addition of agonist produced no further increase in the height of contraction.

When studying the effects of temperature on the response of the vas deferens to an agonist, comparisons

TABLE 1

Concentration of solutions used to construct concentration-response curves

Concentration of stock solution (M)	Volume added (ml)	Final bath Concentration (M)	-log (M) Bath Concentration
7.2×10^{-6}	.140	1.0×10^{-7}	7.0
7.2×10^{-5}	.125	1.0×10^{-6}	6.0
7.2×10^{-4}	.030	3.2×10^{-6}	5.5
7.2×10^{-4}	.095	1.0×10^{-5}	5.0
7.2×10^{-3}	.030	3.2×10^{-5}	4.5
7.2×10^{-3}	.095	1.0×10^{-4}	4.0
7.2×10^{-2}	.030	3.2×10^{-4}	3.5
7.2×10^{-2}	.095	1.0×10^{-3}	3.0

were made between vasa deferentia from the same mouse, one at 37°C, the other at 20°C. To eliminate any artifacts due to variation in the levers, the temperature of the tissue bath-lever systems were alternated between experiments. Usually only one concentration-response curve was measured on each tissue. However, for the determinations of the effect of temperature on the (-)-norepinephrine concentration-response curve after tropolone or cocaine pretreatment, comparisons of responses from paired tissues were made using the third consecutive concentration-response curves.

The first experiments to test the effects of cocaine or tropolone on the concentration-response curves were conducted with norepinephrine as the agonist. Other investigators had reported that the second and third concentration-response curves of the rat vas deferens to norepinephrine tended to be similar and had a greater height of contraction than the first curve on the same tissue (Patil et al., 1967). Therefore, to determine the effect of cocaine or tropolone on the norepinephrine concentration-response curve, the second consecutive concentration-response curve was used as the control and the third curve was obtained in the presence of cocaine or tropolone. After each curve, the tissue was washed five times during a ten minute interval. When no pre-treatment was used, the tissue was allowed to equilibrate for ten minutes after washing. Tropolone or cocaine was

added at the beginning of the equilibration period between the second and third concentration-response curves, and the equilibration period was extended for the appropriate amount of time.

The maximum height of contraction of consecutive concentration-response curves of metaraminol and alpha-methyl norepinephrine tended to decrease (see Appendix). Therefore, in order to determine the effect of cocaine or tropolone on the concentration-response curves of metaraminol, alpha-methyl norepinephrine, and methoxamine, paired vasa deferentia were used. Only one concentration-response curve was measured on each tissue. Both vasa deferentia were maintained at the same temperature; one vas deferens was the control and the other was pretreated with cocaine or tropolone.

The following pretreatments were used to alter the mechanisms for terminating the action of catecholamines: (1) to inhibit MAO, the mice were injected intraperitoneally with 100 mg/kg of pargyline 24 hrs prior to sacrifice (Oppermann, 1970); (2) to inhibit COMT, the tissues were incubated with 3×10^{-5} M tropolone for 30 min preceding the measurement of the concentration-response curve; and (3) to inhibit intraneuronal uptake, the tissues were incubated with 3×10^{-5} M cocaine for 20 min before obtaining the concentration-response curve.

The concentration of 3×10^{-5} M tropolone was selected on the basis of the data presented in Table 2.

TABLE 2

The effect of tropolone on the response of the mouse vas deferens to (-)-norepinephrine

Molar Concentration of Tropolone[a]	°C[b]	-log Molar ED_{50} (-)-norepinephrine with S.E.M.		Log Difference[c]	p[d]	Maximum contraction (cm.)[e] (-)-norepinephrine with S.E.M.		% of control[f]	p[d]	n[g]
		Control	In presence of Tropolone			Control	In presence of Tropolone			
1×10^{-4}	37°C	5.13±.11	4.81±.12	-.32	> .05	3.66±.66	1.16±.32	32	< .001	8
1×10^{-4}	20°C	6.04±.14	5.54±.06	-.50	< .01	5.04±.50	3.34±.27	66	< .01	8
3×10^{-5}	37°C	5.35±.07	5.24±.08	-.11	> .2	5.43±.48	4.41±.57	81	< .01	8
3×10^{-5}	20°C	5.90±.11	5.83±.08	-.07	> .4	6.80±.44	6.32±.48	93	> .1	8

[a] 30 min incubation time.

[b] Bath temperature.

[c] Log difference = (-log molar ED_{50} in the presence of tropolone) - (-log molar ED_{50} of control).

[d] Level of significance of difference between control and tropolone pretreated.

[e] Centimeters of pen deflection.

[f] Maximum contraction in the presence of tropolone/maximum contraction of control x 100.

[g] Number of observations.

The concentration of 1×10^{-4} M tropolone significantly decreased the potency of (-)-norepinephrine at 20°C and in addition, decreased the maximum contraction at both 20°C and 37°C. Similar results have been reported by Kalsner and Nickerson (1969a) and Krell and Patil (1972). The concentration of 3×10^{-5} M tropolone had less of a depressant effect upon the tissue, but the maximum height of contraction at 37°C was still significantly decreased by 3×10^{-5} M tropolone pretreatment.

The determination of which concentration of cocaine to use was based on the data in Table 3. The concentration of 3×10^{-5} M was used because it altered the potency of (-)-norepinephrine more than the other concentrations of cocaine tested. However, 1×10^{-4} M cocaine tended to have a greater effect on the degree of maximum contraction.

Statistical analysis of the concentration-response curves

Two parameters of each concentration-response curve were measured, the -log molar ED_{50} and the maximum height of contraction. The -log molar ED_{50} was calculated using the maximum response of each tissue to the agonist as 100%. When the comparison of the -log molar ED_{50} values was made between paired vasa deferentia or between consecutive concentration-response curves on the same vas deferens, a two-tailed test for "comparison of sample

TABLE 3

The effect of cocaine on the response of the mouse vas deferens to (-)-norepinephrine at 37°C

Molar Concentration of Cocaine[a]	-log Molar ED_{50} (-)-norepinephrine with S.E.M.		Log Difference[b]	P[c]	Maximum contraction (cm)[d] (-)-norepinephrine with S.E.M.		% of Control[e]	P[c]	n[f]
	Control	In presence of cocaine			Control	In presence of cocaine			
1×10^{-5}	$5.27 \pm .11$	$5.58 \pm .13$	.31	< .01	$5.93 \pm .63$	$6.01 \pm .65$	101	-	14
3×10^{-5}	$5.38 \pm .11$	$6.02 \pm .12$	.68	< .001	$5.19 \pm .40$	$6.27 \pm .54$	121	< .001	23
1×10^{-4}	$5.51 \pm .25$	$5.99 \pm .17$	.48	< .02	$5.03 \pm .45$	$7.55 \pm .81$	150	< .01	9

[a] 20 min incubation time.

[b] Log difference = (-log molar ED_{50} in the presence of cocaine) - (-log molar ED_{50} of control).

[c] Level of significance of difference between control and cocaine pretreated.

[d] Centimeters of pen deflection.

[e] Maximum contraction in the presence of cocaine/maximum contraction of control x 100.

[f] Number of observations.

means; paired observations" was used (Steel and Torrie, 1960). When P was less than 0.05, the difference was considered statistically significant.

The maximum height of contraction of the vasa deferentia were illustrated graphically by plotting the centimeters of contraction versus the -log molar concentration of agonist. When the comparison of the differences in centimeters of maximum contraction was made between paired vasa deferentia or consecutive responses on the same vas deferens, the statistical analysis was the same as above.

When comparisons of the differences in -log molar ED_{50} values or mean maximum heights of contraction were made for non-paired tissues, an F test was performed to determine if the variances could be pooled. If the variances were pooled, the test for "comparison of two sample means; unpaired observations, equal variances" was used (Steel and Torrie, 1960). If the variances were not pooled the test for "comparison of sample means for unpaired observations and unequal variances" was used (Steel and Torrie, 1960). The level of significance was $P < 0.05$.

Determination of the retention of ^{14}C-norepinephrine

The vasa deferentia were dissected as described above. The tissue was placed in 1 ml of the physiological

salt solution described previously and was allowed to equilibrate for twenty minutes. The temperature of the incubation mixture was maintained at either 37°C or 20°C and aerated with 95% O_2 and 5% CO_2.

The tissues were incubated for 10 min with either 3.47×10^{-6} M 7-^{14}C-(±)-norepinephrine(±)-bitartrate (specific activity = 27.6 mCi/mM) or 2.69×10^{-6} M 8-^{14}C-(-)-norepinephrine(+)-bitartrate (specific activity = 57 mCi/mM). The concentration of ^{14}C-norepinephrine used was the mean of the ED_{50} values of each agonist at 20°C and 37°C. After incubation, the tissue was transferred to 5 ml of aerated, drug-free, physiological salt solution and washed two times in a 10 min period.

After the rinse period, the vas deferens was blotted and weighed to the nearest tenth of a milligram. The tissue was immediately placed in a scintillation vial containing 0.25 ml of Soluene 100 (Packard) and allowed to digest overnight. An aliquot of the bath medium was treated in a similar manner. After digestion, ten milliliters of Insta-Gel (Packard) was added to each vial and the vials were placed in the dark for 12 hrs. The activity in each vial was determined with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 2002 (window 50-1000, gain 6). The counting efficiency was determined by the internal standard method.

The tissue/medium ratio was calculated by comparing the dpm per gram wet weight of tissue to the dpm per milliliter of bath medium. The statistical significance of the difference in retention at the two temperatures was determined by the test for paired observations, as described in the section on analysis of the concentration-response curves.

Chemicals and solutions

The drugs used in this study were: (-)-norepinephrine, (±)-norepinephrine, tropolone (Regis Chemical Co.); (-)-norepinephrine bitartrate, (-)-alpha-methyl norepinephrine, and (-)-metaraminol(+)-bitartrate (courtesy of Sterling-Winthrop Research Institute); (±)-alpha-methyl norepinephrine hydrochloride and (-)-phenylephrine hydrochloride (Mann Research Laboratories, Inc.); (±)-methoxamine hydrochloride (courtesy of Burroughs Wellcome and Co.); pargyline hydrochloride (courtesy of Abbott Laboratories); (±)-metaraminol bitartrate (Merck Sharpe and Dohme Research Laboratories); cocaine hydrochloride (Mallinckrodt Chemical Works); 7-^{14}C-(±)-norepinephrine(±)-bitartrate, 8-^{14}C-(-)-norepinephrine(+)-bitartrate (Amersham Searle). Other chemicals and reagents were analytical grade.

All drug and chemical solutions were prepared with deionized distilled water. Dissolution of the free bases of (-)-norepinephrine, (±)-norepinephrine and (-)-alpha-

methyl norepinephrine was aided by adding drops of 1 N HCl until the compounds dissolved in a 0.05% solution of sodium metabisulfite. The ^{14}C-norepinephrine compounds were dissolved in 0.01 N HCl containing 0.05% sodium metabisulfite and stored at 0-4°C. All other drugs were dissolved in 0.05% sodium metabisulfite. Solutions of all drugs except ^{14}C-norepinephrine were prepared fresh daily.

RESULTS

Effects of temperature on the response of the mouse vas deferens to norepinephrine

The isolated mouse vas deferens was more sensitive to contractile effects of (-)-norepinephrine at 20°C than at 37°C, as illustrated in Figure 1. This change in sensitivity resulted in a 0.70 log unit increase in the -log ED_{50} and a 27% increase in the maximum degree of contraction (Table 4). Although not quantified, the response of the vas deferens to norepinephrine at 20°C developed more slowly than the response at 37°C. In addition, the time required for the lever to return to the base line level during the wash period was longer at 20°C than at 37°C.

Since Oppermann et al. (1972) and Muñoz-Ramirez et al. (1973a) suggest that increases in potency of adrenergic agonists may be brought about by alteration of disposition mechanisms, a systematic study of the effects of temperature on activities of a number of agonists was performed. The structures of these compounds are illustrated in Table 5. In addition, the effects of various drug treatments on activities of these agonists were determined in order to aid elucidation of mechanisms responsible for temperature-dependent sensitivity changes.

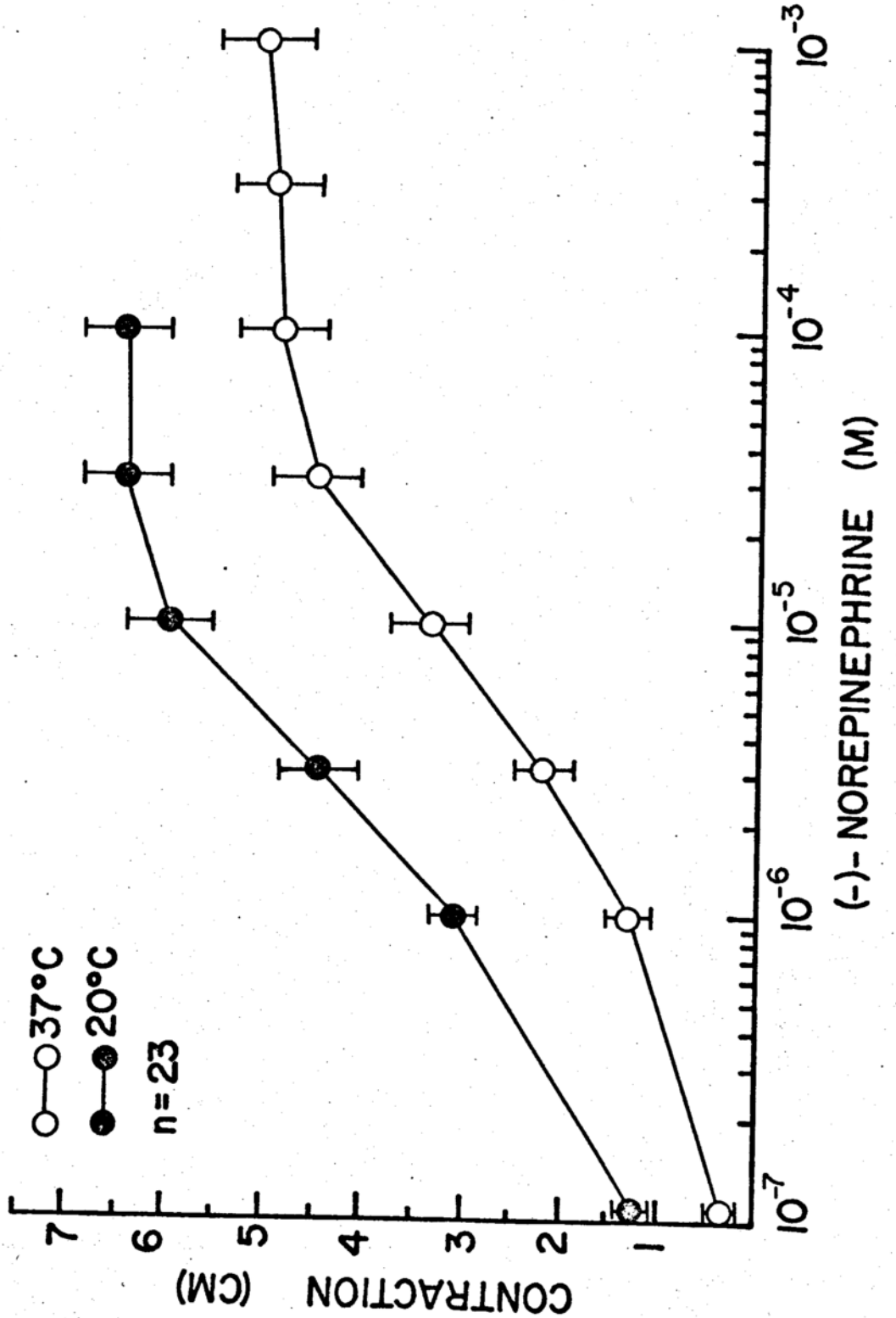

37°C
20°C
n=23
CONTRACTION (CM)
1
2
3
4
5
6
7
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
(-)- NOREPINEPHRINE (M)

TABLE 4

Effects of temperature on the response of the mouse vas deferens to norepinephrine and methoxamine

Agonist	-log Molar ED_{50} with S.E.M.		Log Difference[a]	P[b]	Maximum Contraction (cm)[c] with S.E.M.		% of	P[b]	n[e]
	37°C	20°C			37°C	20°C	37°C[d]		
(-)-Norepinephrine Curve #1	5.32±.08	6.02±.07	.70	<.001	5.13±.46	6.52±.44	127	<.05	23
(-)-Norepinephrine[f] Curve #2	5.28±.04	5.86±.06	.58	<.001	5.11±.39	6.08±.34	119	<.01	32
(±)-Methoxamine	5.03±.09	4.86±.03	-.17	>.05	3.29±.45	5.48±.60	167	<.001	16

[a]Log difference = (-log molar ED_{50} at 20°C) - (-log molar ED_{50} at 37°C).

[b]P = level of significance of difference between values obtained at 37°C and 20°C.

[c]Centimeters of pen deflection.

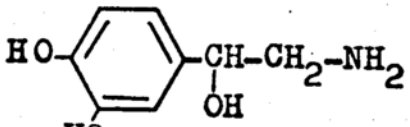
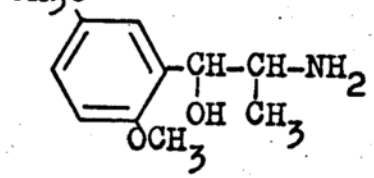
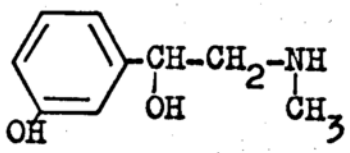
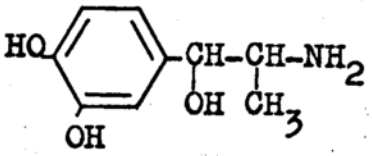
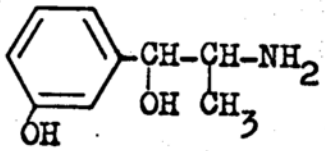
[d]Maximum contraction at 20°C/maximum contraction at 37°C x 100.

[e]Number of observations.

[f]Second consecutive concentration - response curve to norepinephrine obtained from the same tissue.

TABLE 5

Chemical structures of alpha adrenergic agonists used in present studies

Compound	Structural Formula
Norepinephrine	
Methoxamine	
Phenylephrine	
alpha-Methyl-norepinephrine	
Metaraminol	

The importance of the mechanisms responsible for termination of the action of sympathomimetic amines in temperature-dependent sensitivity changes

To examine the hypothesis that the shift to the left of the norepinephrine concentration-response curve was due to an inhibition of one of the means of norepinephrine inactivation, the effect of temperature on the methoxamine concentration-response relationship was determined. Methoxamine is a direct acting alpha receptor agonist which has a low affinity for the intraneuronal uptake mechanism and is not metabolized by MAO or COMT (Iversen, 1967; Trendelenburg et al., 1970). If the potency of methoxamine were not affected by temperature changes, it would suggest that an inhibition of at least one of the major mechanisms of norepinephrine inactivation, rather than a tissue or receptor change, was responsible for the increased potency of (-)-norepinephrine at 20°C. As noted in Figure 2 and also in Table 4, the -log molar ED_{50} of methoxamine was not affected by temperature changes; however, the maximum response to methoxamine was greater at 20°C than at 37°C.

The results in Table 6 demonstrate that the maximum height of contraction of the mouse vas deferens varied significantly between different sets of experiments. The maximum contractions in response to methoxamine in January 1972 were significantly greater than in June

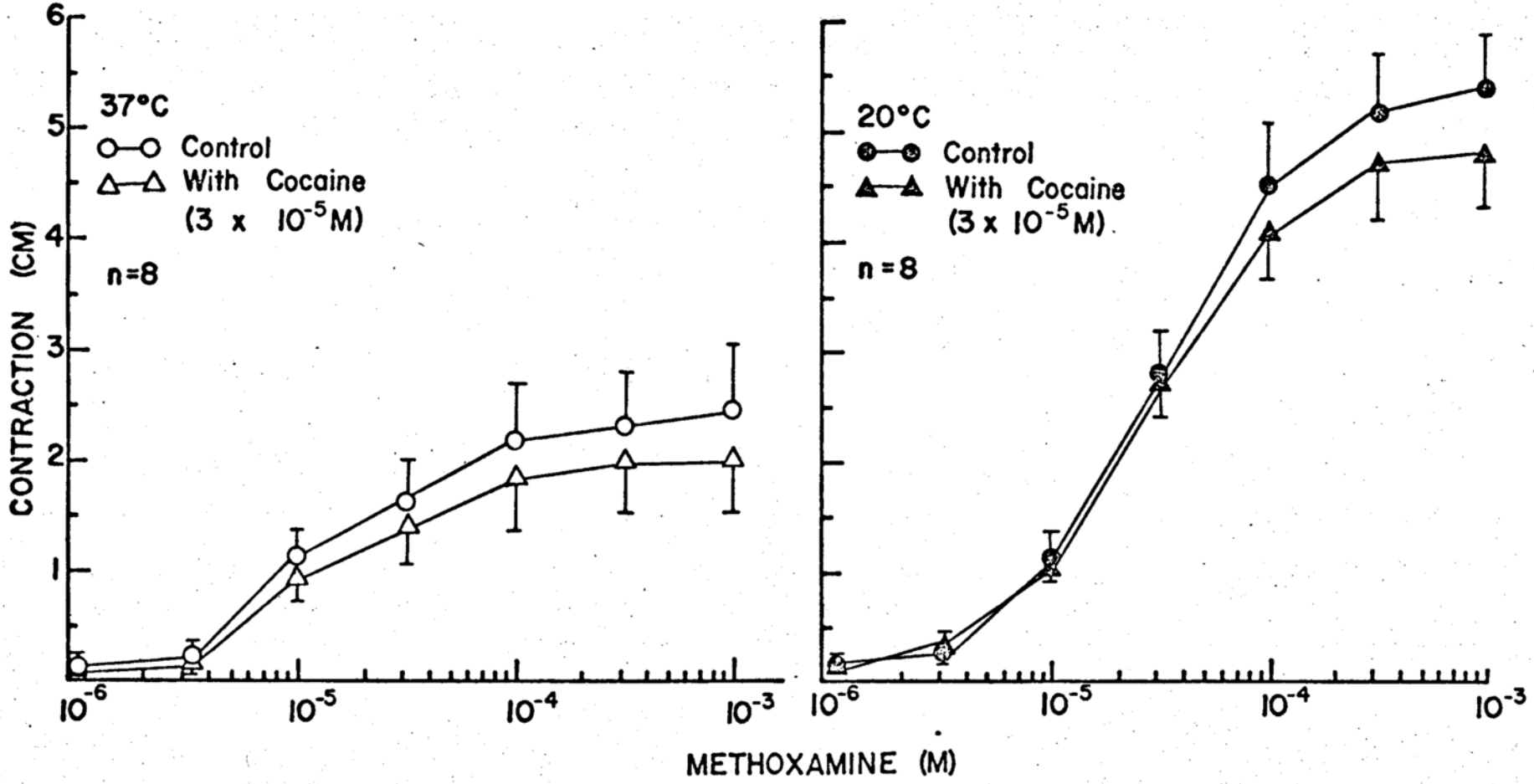

37°C
Control
With Cocaine
(3 x 10⁻⁵M)
n=8
20°C
Control
With Cocaine
(3 x 10⁻⁵M)
n=8
CONTRACTION (CM)
METHOXAMINE (M)
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
1
2
3
4
5
6

TABLE 6

Responses of the mouse vas deferens to norepinephrine and methoxamine on different dates

Agonist	Date	-log Molar ED_{50} with S.E.M. 37°C	-log Molar ED_{50} with S.E.M. 20°C	Log Difference[a]	P[b]	Maximum Contraction (cm)[c] with S.E.M. 37°C	Maximum Contraction (cm)[c] with S.E.M. 20°C	% of 37°C[d]	P[b]	n[e]
(±)-Methoxamine	1/72	5.00±.07	4.84±.04	-.16	>.05	4.27±.57	7.2 ±.76	169	<.001	8
(±)-Methoxamine	6/72	5.06±.17	4.87±.04	-.19	>.2	2.31±.52	3.77±.31	163	<.05	8
(-)-Norepinephrine	5/72	5.15±.14	6.04±.14	.89	<.01	3.87±.58	5.70±.55	147	<.05	8
(-)-Norepinephrine	5/72	5.36±.15	5.98±.15	.62	<.01	6.46±1.11	5.83±.86	90	>.5	7
(-)-Norepinephrine	10/72	5.44±.12	6.03±.09	.59	<.02	5.21±.45	7.93±.66	152	<.01	8

[a]Log difference = (-log molar ED_{50} at 20°C) - (-log molar ED_{50} at 37°C).

[b]P = level of significance of difference between 37°C and 20°C.

[c]Centimeters of pen deflection.

[d]Maximum contraction at 20°C/maximum contraction at 37°C x 100.

[e]Number of observations.

1972 at both temperatures studied (37°C, $P < .05$; 20°C, $P < .01$). In addition, with (-)-norepinephrine as the agonist, the maximum height of contraction in October 1972 was significantly greater than the maximum contraction in May 1972 at 20°C ($P < .05$) but not at 37°C. In general, the ratio of the maximum height of contraction at 20°C to the maximum height of contraction at 37°C in paired preparations did not vary between series of experiments. However, the ratio of maximum heights of contraction of paired preparations to norepinephrine at 20°C and 37°C measured in May 1972 was significantly less ($P < .05$) than the ratio of maximum heights of contraction measured in October 1972. The values of the -log molar ED_{50} and the log differences between 37°C and 20°C were more consistent between series of experiments.

In light of these results, very little significance should be placed on absolute degree of contractions measured at different times of the year or in non-paired preparations. A similar variation in the degree of maximum contraction of the vas deferens has not been mentioned by other investigators. However, Westfall et al. (1972) noted that the degree of displacement of the norepinephrine and histamine concentration-response curves of the guinea-pig vas deferens induced by decentralization was 29-fold and 15-fold, respectively, in one series of experiments, while in another series of

experiments, the corresponding values were 2.5-fold and 2-fold. They suggested that the variations were due to housing in the presence or absence of female guinea-pigs. Due to the variability of the conditions of our animal room at the time, it was not possible to determine if the housing conditions affected the maximum degree of contraction of the mouse vas deferens.

Relationship between the activity of COMT and temperature-dependent sensitivity changes

Experiments were designed to determine if a decrease in the activity of COMT was responsible for the increased sensitivity of the mouse vas deferens to norepinephrine at 20°C. As noted in Table 7, the difference between the values of the -log molar ED_{50} for norepinephrine at 20°C and at 37°C was the same in the absence or presence of tropolone, 3×10^{-5} M. The results obtained with phenylephrine, a direct-acting alpha receptor agonist which is not a substrate for COMT are shown in Table 7. Lowering the temperature from 37°C to 20°C resulted in a 0.37 log unit shift of the concentration-response curve to the left and a slight but insignificant increase in the maximum response.

TABLE 7

Effect of temperature on the response of the mouse vas deferens to adrenergic agonists in the absence of the influence of COMT

Agonist	Pretreatment	-log Molar ED_{50} with S.E.M. 37°C	-log Molar ED_{50} with S.E.M. 20°C	Log Difference[a]	P[b]	Maximum Contraction (cm)[c] with S.E.M. 37°C	Maximum Contraction (cm)[c] with S.E.M. 20°C	% of 37°C[d]	P[b]	n[e]
(-)-Norepinephrine[f]	None	5.35±.07	5.90±.11	.55	<.01	5.43±.48	6.80±.44	125	<.01	8
(-)-Norepinephrine	Tropolone[g]	5.24±.08	5.83±.08	.58	<.01	4.41±.57	6.32±.48	143	<.001	8
(-)-Phenylephrine	None	5.25±.09	5.62±.10	.37	<.05	3.20±.59	3.78±.49	118	>.5	8

[a]Log difference = (-log molar ED_{50} at 20°C) - (-log molar ED_{50} at 37°C).

[b]P = level of significance of difference between 37°C and 20°C.

[c]Centimeters of pen deflection.

[d]Maximum contraction at 20°C/maximum contraction at 37°C x 100.

[e]Number of observations.

[f]Second consecutive concentration - response curve of norepinephrine. Measured before tropolone pretreatment.

[g]3×10^{-5} M for 30 min.

Relationship between the activity of MAO and temperature-dependent sensitivity changes

Inhibition of MAO by pargyline did not prevent the increase in sensitivity of the vas deferens to (-)-norepinephrine or (-)-phenylephrine at 20°C. The responses of the vasa deferentia taken from mice pretreated with pargyline are illustrated in Figure 3 and summarized in Table 8. The (-)-norepinephrine concentration-response curve at 20°C was 0.78 log units to the left of the curve obtained at 37°C and the maximum response was greater at 20°C than at 37°C. With (-)-phenylephrine as the agonist, there was a 0.72 log unit shift to the left at 20°C. The degree of shift to the left of the concentration-response curve was not significantly greater from that obtained without pargyline pretreatment. However, the absolute degree of contraction in response to phenylephrine at 20°C after pargyline pretreatment was significantly greater than the absolute degree of contraction without pargyline ($P < .05$).

Additional data concerning the role of MAO were obtained by determining the responses of the vas deferens to alpha-methyl norepinephrine, an alpha receptor agonist which is not metabolized by MAO. The first experiments were conducted with the racemic form

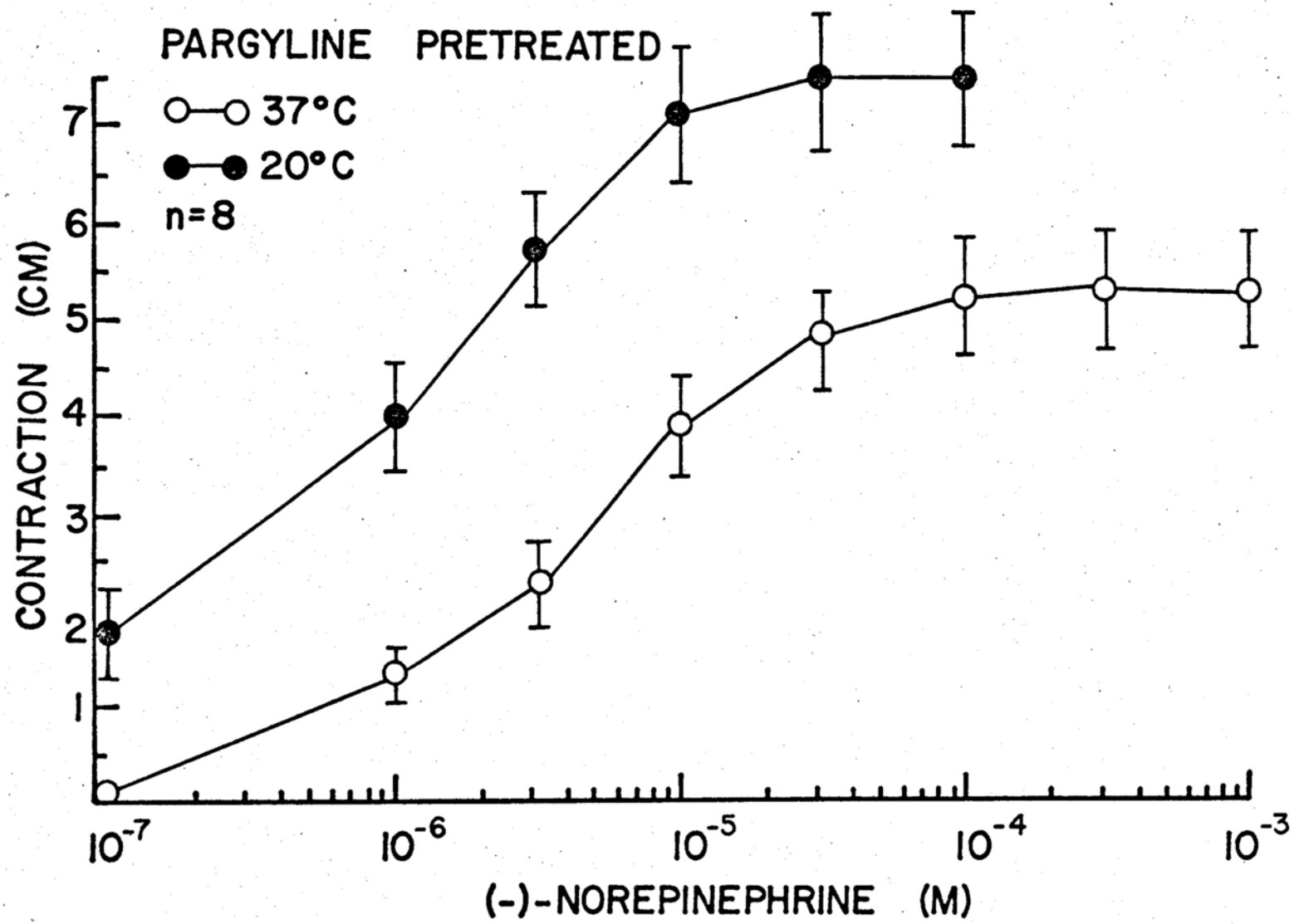

PARGYLINE PRETREATED
37°C
20°C
n=8
CONTRACTION (CM)
1
2
3
4
5
6
7
10-7
10-6
10-5
10-4
10-3
(-)-NOREPINEPHRINE (M)

TABLE 8

Effect of temperature on the response of the mouse vas deferens to adrenergic agonists in the absence of the influence of MAO

Agonist	Pretreatment	-log Molar ED_{50} with S.E.M.		Log Difference[a]	P[b]	Maximum Contraction (cm)[c] with S.E.M.		% of	P[b]	n[e]
		37°C	20°C			37°C	20°C	37°C[d]		
(-)-Norepinephrine	Pargyline[f]	5.42±.09	6.20±.16	.78	<.01	5.33±.60	7.62±.70	143	<.05	8
(-)-Phenylephrine	Pargyline[f]	5.27±.19	5.99±.23	.72	<.05	3.20±.66	5.19±.33	162	>.05	8
(±)-α-Methyl norepinephrine	None	4.69±.07	4.64±.10	-.05	>.5	5.37±.96	5.37±.48	100	-	8
(-)-α-Methyl norepinephrine	None	5.11±.05	5.29±.07	.18	<.02	6.43±.42	7.10±.62	110	>.1	11

[a]Log difference = (-log molar ED_{50} at 20°C) - (-log molar ED_{50} at 37°C).

[b]P = level of significance of difference between 37°C and 20°C.

[c]Centimeters of pen deflection.

[d]Maximum contraction at 20°C/maximum contraction at 37°C x 100.

[e]Number of observations.

[f]100 mg/kg 24 hrs prior to sacrifice.

of the compound. The -log molar ED_{50} and the maximum response to (±)-alpha-methyl norepinephrine were not affected by changes in bath temperatures (Table 8). Since the dextro-rotatory form of some compounds is known to interfere with the neuronal uptake of the levo-rotatory form (Patil et al., 1970), studies were also conducted with the levo-rotatory isomer. As noted in Figure 4 and Table 8, the -log molar ED_{50} of (-)-alpha-methyl norepinephrine was 0.18 log units larger at 20°C than at 37°C. The maximum response at 20°C was not significantly different from the maximum at 37°C.

Studies to determine the importance of neuronal uptake in temperature-dependent sensitivity changes

The first set of experiments was designed to measure the responses of the vas deferens at 37°C and 20°C when uptake was the only mechanism involved in terminating the activity of the agonist. This was accomplished by measuring the response to (-)-phenylephrine after pargyline pretreatment, the response to (-)-alpha-methyl norepinephrine after tropolone pretreatment and the response to metaraminol alone. Metaraminol was used because it is not metabolized by MAO or COMT; however, it can release norepinephrine in addition to acting directly on the receptor. The results from these studies are presented in Table 9.

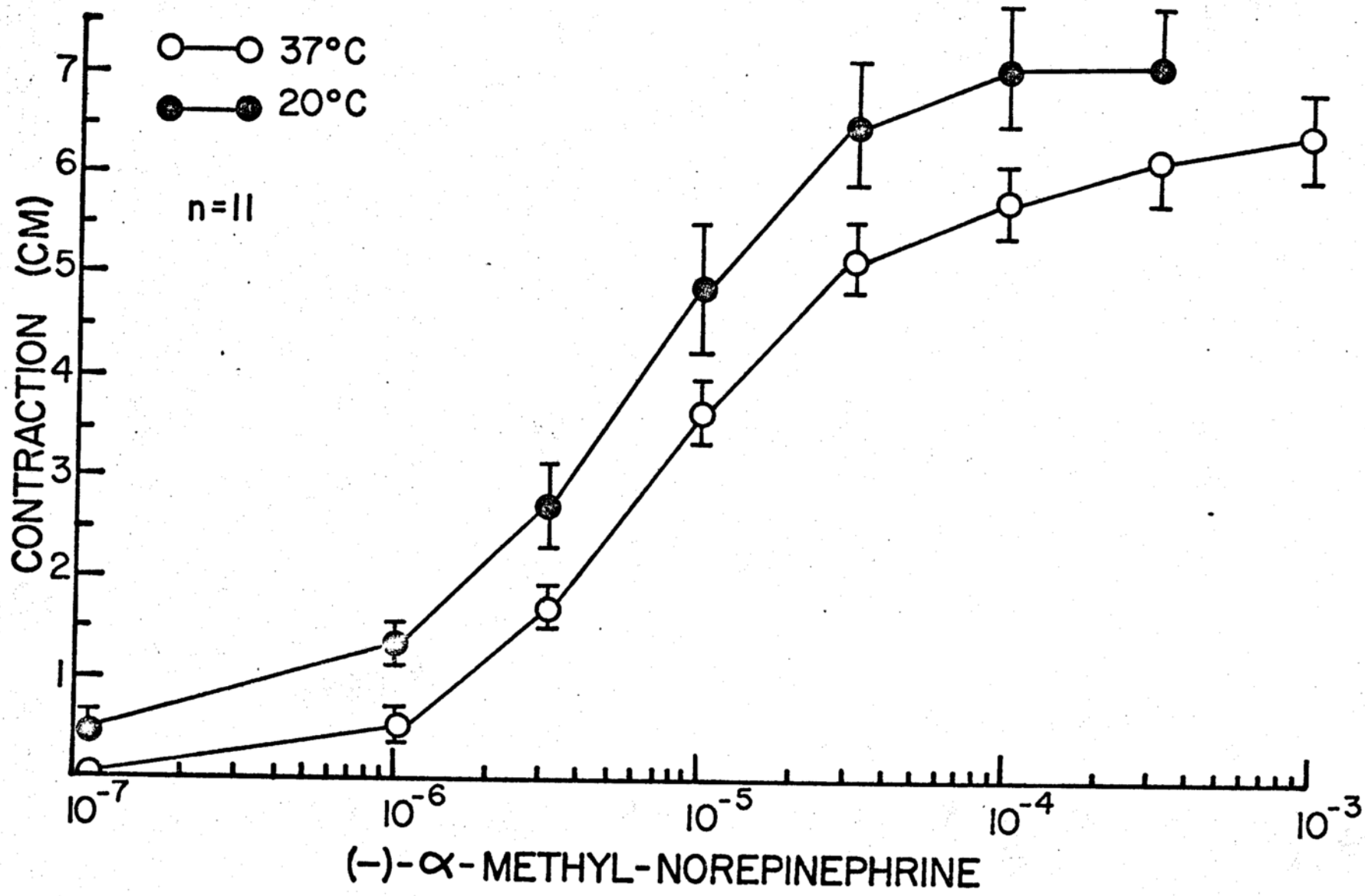

37°C
20°C
n=11
CONTRACTION (CM)
1
2
3
4
5
6
7
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
(−)-α-METHYL-NOREPINEPHRINE

TABLE 9

Effect of temperature on the responses of the mouse vas deferens to adrenergic agonists in the absence of the influence of COMT and MAO

Agonist	Pretreatment	-log Molar ED_{50} with S.E.M.		Log Difference[a]	P[b]	Maximum Contraction (cm)[c] with S.E.M.		% of 37°C[d]	P[b]	n[e]
		37°C	20°C			37°C	20°C			
(-)-Phenylephrine	Pargyline[f]	5.27±.19	5.99±.23	.72	<.05	3.20±.66	5.19±.33	162	>.05	8
(-)-α-Methyl norepinephrine	Tropolone[g]	5.00±.09	5.19±.06	.19	>.1	5.12±.79	6.16±.59	120	>.1	12
(±)-Metaraminol	None	4.64±.07	4.54±.06	-.11	>.3	7.55±.46	6.12±.58	81	<.05	8
(-)-Metaraminol	None	4.76±.07	4.82±.10	.06	>.5	6.30±.35	5.90±.45	94	>.5	8
(-)-Phenylephrine	Pargyline[f] Cocaine[h]	5.74±.28	5.85±.14	.11	>.5	7.56±.84	6.20±.45	82	>.2	8

[a]Log difference = (-log molar ED_{50} at 20°C) - (-log molar ED_{50} at 37°C).

[b]P = level of significance of difference between 37°C and 20°C.

[c]Centimeters of pen deflection.

[d]Maximum contraction at 20°C/maximum contraction at 37°C x 100.

[e]Number of observations.

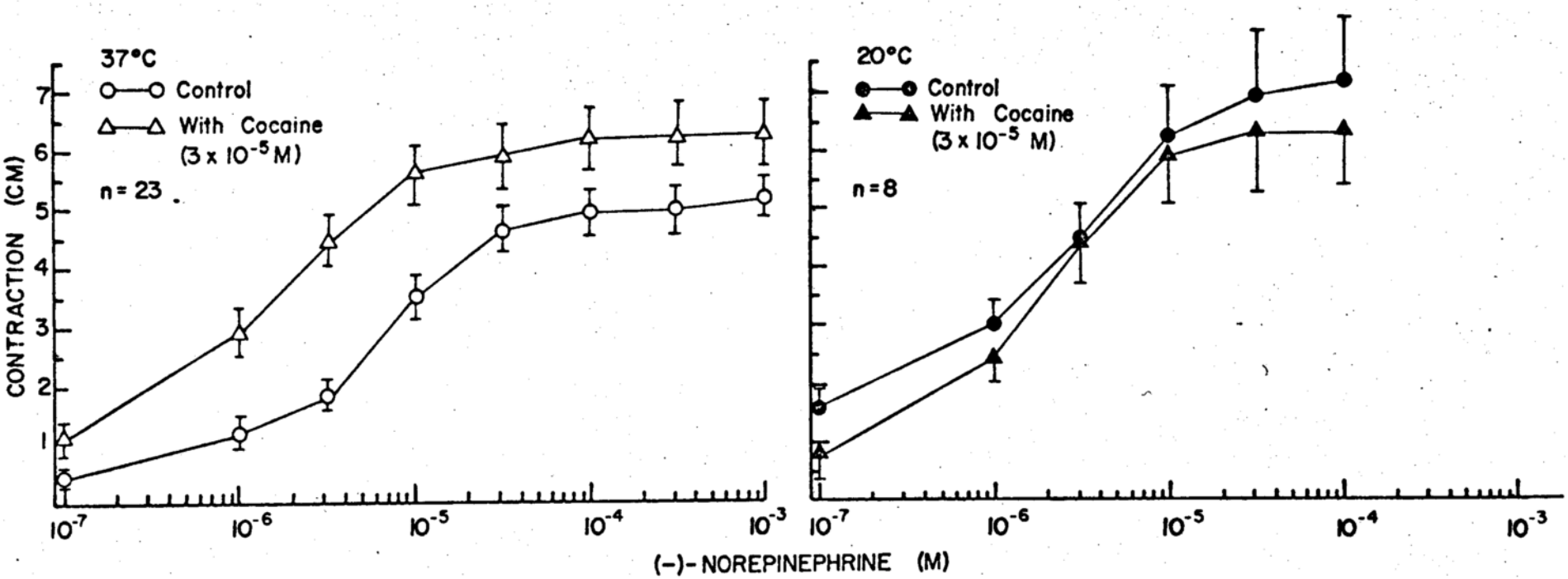
[f]100 mg/kg 24 hrs prior to sacrifice.

[g]3×10^{-5} M for 30 min.

[h]3×10^{-5} M for 20 min.

As stated previously, after pargyline pretreatment, the vas deferens was more sensitive to (-)-phenylephrine at 20°C than at 37°C. After tropolone pretreatment, (-)-alpha-methyl norepinephrine also tended to be more potent at 20°C than at 37°C but the 0.19 log unit difference was not statistically significant. The potencies of racemic and levo-rotatory metaraminol were not significantly affected by the change in temperature. In addition, the maximum contractions produced by (-)-alpha-methyl norepinephrine and metaraminol were not significantly affected by changes in temperature.

In the second set of experiments the responses of the vasa deferentia to various agonists were measured at 20°C and 37°C after pretreatment with cocaine, an inhibitor of intraneuronal uptake (Iversen and Langer, 1969). As illustrated in Figure 5, at 37°C the concentration-response curve of (-)-norepinephrine obtained in the presence of cocaine (3×10^{-5} M) was 0.68 log units to the left of the control response and the maximum height of contraction was increased by 21%. At 20°C the -log molar ED_{50} was not affected by cocaine and the maximum response was decreased by 12% (Table 10). In addition, after cocaine pretreatment, the values of the -log molar ED_{50} of (-)-norepinephrine at 20°C and at 37°C were equivalent. Cocaine did not increase the lag time between the introduction of the agonist into the bath and the time of the response.


37°C
Control
With Cocaine
(3 x 10⁻⁵ M)
n = 23
20°C
Control
With Cocaine
(3 x 10⁻⁵ M)
n = 8
CONTRACTION (CM)
1
2
3
4
5
6
7
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
(-)- NOREPINEPHRINE (M)

The studies of the effect of cocaine on (-)-phenylephrine concentration-response curves were conducted on vasa deferentia from mice that had been pretreated with pargyline (Table 9). After cocaine pretreatment, the values of the -log molar ED_{50} of (-)-phenylephrine at 37°C and 20°C were not significantly different. In addition, from paired preparations the maximum degree of contraction at 37°C was not significantly different from the maximum contraction at 20°C. Data from unpaired preparations showed that cocaine pretreatment significantly (P .01) enhanced the maximum responses produced by phenylephrine (Table 9).

The effects of cocaine on concentration-response curves of (-)-alpha-methyl norepinephrine at 37°C, of (±)-methoxamine at 20°C and 37°C (Figure 2) and of (-)-metaraminol at 37°C were also measured (Table 10). Cocaine pretreatment at 37°C caused a shift to the left of the (-)-alpha-methyl norepinephrine concentration-response curve but had no effect on the maximum degree of contraction. Cocaine did not significantly affect the values of the -log molar ED_{50} or maximum degree of contraction to (±)-methoxamine or (-)-metaraminol.

The third set of experiments to determine the role of intraneuronal uptake in temperature-dependent sensitivity changes was designed to measure the degree of retention of ^{14}C-norepinephrine by the mouse vas deferens. From data in Table 11, it is apparent that

TABLE 11

Influence of temperature on the retention of norepinephrine by mouse vas deferens

Compound	Bath concentration (x 10^{-6} M)	T/M[a] with S.E.M. 37°C	T/M[a] with S.E.M. 20°C	P[b]	n[c]
^{14}C-(±)-Norepinephrine	3.5	2.13±.20	.97±.06	<.01	8
^{14}C-(±)-Norepinephrine[d]	3.5	2.44±.06	1.02±.05	<.001	7
^{14}C-(-)-Norepinephrine[d]	2.7	2.42±.20	.96±.06	<.001	8
^{14}C-(±)-Norepinephrine[d,e]	3.5	1.16±.17	.82±.04	>.05	6

[a] T/M = dpm per gram wet weight of tissue/dpm per ml of bath medium. Tissue incubated for 10 min with amine and washed for 10 min.

[b] P = level of significance of difference of 37°C and 20°C.

[c] Number of observations.

[d] Obtained in the presence of 3 x 10^{-5} M tropolone for 30 min. Pretreated with pargyline, 100 mg/kg, 24 hrs prior to sacrifice.

[e] Obtained in the presence of cocaine 3 x 10^{-5} M for 20 min.

the tissue/medium ratios of both the racemic and levo-rotatory forms of ^{14}C-norepinephrine were lower at 20°C than at 37°C. Pretreatment with tropolone and pargyline had very little effect on the tissue/medium ratios; however, cocaine pretreatment significantly reduced the retention at both 37°C and 20°C. The tissue/medium ratios for paired vasa deferentia at 37°C and 20°C were not significantly different after cocaine pretreatment.

DISCUSSION

Alteration of potency

The -log molar ED_{50} and the maximum contraction of the mouse vas deferens in response to (-)-norepinephrine were significantly greater at 20°C than at 37°C. In contrast, the potencies of methoxamine at 20°C and 37°C were very similar, a finding which suggests that the increase in the potency of (-)-norepinephrine at 20°C was due to an alteration of one of the mechanisms for terminating the action of catecholamines. A comparison of the concentration-response curves of (-)-norepinephrine at 20°C and 37°C, before and after cocaine pretreatment, suggests that inhibition of neuronal uptake at 20°C was responsible for the temperature-dependent changes in the potency of norepinephrine. Without cocaine pretreatment the -log molar ED_{50} for (-)-norepinephrine at 20°C was significantly different from the -log molar ED_{50} at 37°C. After neuronal uptake was inhibited by cocaine, the values of the -log molar ED_{50} at 20°C and 37°C were equivalent. This evidence that temperature-dependent changes in the potency of (-)-norepinephrine were due to inhibition of neuronal uptake at 20°C is supported by the data obtained with phenylephrine and cocaine. After cocaine pretreatment, the potency of phenylephrine on vasa deferentia from

pargyline pretreated mice was not altered by changes in bath temperature.

The conclusion is further substantiated by the studies of the effect of temperature and cocaine on the retention of ^{14}C-norepinephrine by the mouse vas deferens. The studies of the retention of ^{14}C-norepinephrine were designed to measure the differences in intraneuronal uptake at 20°C and 37°C; however, some norepinephrine may have been retained extraneuronally. The mouse vas deferens, unlike the rat vas deferens, retains norepinephrine extraneuronally after two minutes of washing in norepinephrine-free solution (Burnstock et al., 1972). The level of norepinephrine retained extraneuronally in the mouse vas deferens after ten minutes of washing has not been determined, but it is possible that a small amount of the retained norepinephrine was located extraneuronally. However, cocaine reduced the tissue/medium ratio of ^{14}C-norepinephrine at 37°C to a value that was not significantly different from the tissue/medium ratio at 20°C. This suggests that a difference in the capacity for intraneuronal uptake was the major reason for the difference in the tissue/medium ratios of paired vasa deferentia at 37°C and 20°C.

Experiments also were designed to determine the role of MAO and COMT in temperature-dependent sensitivity

changes. The data indicate that the difference in the potency of norepinephrine at 20°C and 37°C was not due to a decrease in the activity of these enzymes at 20°C. The evidence for the lack of involvement of COMT in temperature-dependent supersensitivity was derived from the measurements at 20°C and 37°C of the responses of the vas deferens to (-)-phenylephrine and to (-)-norepinephrine after tropolone pretreatment. In both experiments, reducing the temperature to 20°C resulted in a shift to the left of the concentration-response curve. The conclusion stated above is supported by the evidence that cocaine alone could prevent the change in potency of norepinephrine.

The lack of involvement of the activity of COMT in temperature-dependent sensitivity changes in the mouse vas deferens was in direct contrast to its role in the mouse atria. In the atria, the alteration in the activity of COMT was the primary reason for the temperature-dependent supersensitivity to sympathomimetic amines (Muñoz-Ramírez, 1973). In the present study, the activity of COMT was not measured; therefore, it is possible that the activity of COMT was reduced but that this change in enzyme activity was not influential in the temperature-dependent supersensitivity. The potency of (-)-norepinephrine in the vas deferens ($-\log ED_{50}$ = 5.58 at 37°C in the presence of 1×10^{-5} M cocaine) is lower than in the atria ($-\log ED_{50}$ = 6.63 in the

presence of 1×10^{-5} M cocaine). As noted by Trendelenburg (1972c) a blockade of COMT results in supersensitivity only in tissues where the -log ED_{50} of the catecholamine is greater than 6.0, because at higher concentrations of catecholamines the activity of COMT is saturated. Therefore, in tissues which are less sensitive to exogenous norepinephrine, COMT only affects a small portion of the total amount of catecholamine.

The conclusion that inhibition of MAO was not responsible for the temperature-dependent supersensitivity is based on the responses of the vas deferens from pargyline-pretreated mice to (-)-norepinephrine and (-)-phenylephrine. In the absence or presence of pargyline pretreatment, the values of the -log ED_{50} of the mouse vas deferens to (-)-norepinephrine were significantly different at 37°C and 20°C. Similar results were obtained when (-)-phenylephrine was the alpha receptor agonist. This evidence is further supported by the significantly different values of the -log ED_{50} of (-)-alpha-methyl norepinephrine at 20°C and 37°C.

Although the difference was not statistically significant, pargyline pretreatment tended to shift the concentration-response curve to (-)-phenylephrine at 20°C a greater degree to the left than it shifted the curve at 37°C. This suggests that the activity of MAO was more important in terminating the action of

(-)-phenylephrine at 20°C than at 37°C. This agrees with previous observations in the rabbit aorta that MAO is important in the inactivation of phenylephrine (Kalsner and Nickerson, 1968). Since phenylephrine is not a substrate for COMT and since intraneuronal uptake is inhibited at 20°C, MAO may be more important in terminating the action of phenylephrine at 20°C than at 37°C.

In contrast to the responses to norepinephrine, the potencies of (±)-alpha-methyl norepinephrine, (±)-metaraminol and (-)-metaraminol were not greater at 20°C than at 37°C. There are several possible factors which could result in the lack of shift of the concentration-response curves of these compounds. First, the potencies of the compounds were low; therefore, the capacity for neuronal uptake may have been saturated before the ED_{50} concentration was reached. Second, in the case of (±)-metaraminol and (±)-alpha-methyl norepinephrine, the dextro-rotatory isomer may have inhibited the uptake of the levo-rotatory isomer. This has been demonstrated by Patil et al. (1970). Third, the inhibition of neuronal uptake by reducing the temperature to 20°C may have prevented metaraminol or alpha-methyl norepinephrine from releasing norepinephrine from the neuronal vesicles.

In general, the data from the studies of the effect of temperature on the potency of catecholamines suggest that a decrease in neuronal uptake was responsible for

the increased potency of (-)-norepinephrine at 20°C. In addition, the data suggest that neuronal uptake is the primary mechanism for terminating the activity of (-)-norepinephrine in the mouse vas deferens. Previous investigators have shown that decreasing the temperature decreases the activity of MAO and COMT in atrial homogenates (Oppermann et al., 1972). Although the activities of MAO and COMT in homogenates of the mouse vas deferens were not measured it is possible that the activities of these enzymes were decreased at 20°C. However, the finding that after pretreatment with cocaine the potency of (-)-norepinephrine was not affected by a change in temperature, indicates that the concentration of (-)-norepinephrine at the receptor was not substantially altered by any changes in the activity of MAO or COMT. The access of (-)-norepinephrine to neuronal MAO is inhibited by cocaine pretreatment, which could explain the lack of influence of a decrease in MAO. However, pargyline pretreatment did not significantly affect the potencies of (-)-phenylephrine or (-)-norepinephrine at 37°C.

Since the mouse vas deferens has a dense adrenergic innervation with neurons reaching to every muscle cell (see Introduction), the capacity for neuronal uptake is substantial. The importance of neuronal uptake suggested by this study confirms the conclusions of previous investigators who showed that, in densely innervated

tissues, neuronal uptake was the primary mechanism for terminating the activity of exogenous norepinephrine (Trendelenburg, 1972c).

Alteration of the maximum degree of contraction to adrenergic agonists

In addition to altering the potency of (-)-norepinephrine, decreasing the bath temperature from 37°C to 20°C increased the maximum degree of contraction to this agonist. The maximum degree of contraction to (-)-norepinephrine at 37°C was increased also by cocaine pretreatment. Decreased bath temperature and cocaine pretreatment had a similar effect on the maximum contraction of the mouse vas deferens to (-)-norepinephrine, (-)-alpha-methyl norepinephrine, and (-)-metaraminol. However, cocaine had a greater effect than decreased bath temperature on the maximum response to (-)-phenylephrine after pargyline pretreatment. In addition, the maximum response to methoxamine was increased by lowering the bath temperature to 20°C but the maximum response was not increased by cocaine pretreatment.

Reduction of the bath temperature or cocaine pretreatment did not increase the maximum degree of contraction of the vas deferens to (-)-alpha-methyl norepinephrine or to (-)-metaraminol. This lack of influence on the maximum response to these agonists may be a result of an inhibition of the indirect component of

their action. Patil and Jacobowitz (1968) reported that the maximal responses to (-)-alpha-methyl norepinephrine of the rat vas deferens from reserpine pretreated animals was less than the maximal response of vas deferens from untreated animals. These data suggest that (-)-alpha-methyl norepinephrine has an indirect component of action which affects the maximum degree of contraction of the vas deferens to this agonist. Therefore, inhibition of uptake of these agonists by decreased bath temperature or cocaine pretreatment would tend to obscure the effects of these treatments on the maximum response to (-)-alpha-methyl norepinephrine or (-)-metaraminol.

The marked effect of cocaine on the maximum response to (-)-phenylephrine after pargyline pretreatment may have been a result of the experimental design. The comparison of the maximum response of the vas deferens to (-)-phenylephrine after pargyline pretreatment in the presence and absence of cocaine was a comparison between vasa deferentia from different animals, measured on different dates. Since the degree of maximum contraction tended to fluctuate between groups of experiments, the data may not be an accurate reflection of the effect of cocaine on the maximum response to phenylephrine. Decreasing the bath temperature tended to increase the maximum response to (-)-phenylephrine in paired vasa deferentia. Due to the variability of this type of

measurement a larger number of observations is needed to determine if the increase is significant.

The effect of temperature changes and cocaine pretreatment on the methoxamine concentration-response curve was of particular interest. Methoxamine has been used as a tool by previous investigators to determine whether or not cocaine or decentralization produced a postsynaptic change in the tissue. Kalsner and Nickerson (1969c) reported that cocaine (1×10^{-5} g/ml) potentiated the response of the rabbit aortic strips to methoxamine. In contrast, the responses of the isolated cat nictitating membrane to methoxamine were potentiated by decentralization but not by cocaine pretreatment (Trendelenburg et al., 1970). From these data, it was concluded that pretreatment with cocaine may cause a postsynaptic change in the rabbit aorta but not in the cat nictitating membrane. In the present study, decreasing the bath temperature increased the maximum contraction of the vas deferens to methoxamine, which suggests that temperature changes possibly have a postsynaptic effect on the vas deferens. In contrast, the maximum height of contraction of the vas deferens to methoxamine was not significantly affected by pretreatment of the vas deferens with 3×10^{-5} M cocaine, which suggests that a postsynaptic effect is not produced by this concentration of cocaine. However, a 1×10^{-4} M concentration of cocaine could possibly have

a postsynaptic effect. In preliminary studies the potency of (-)-norepinephrine was affected equally by the 1×10^{-4} M and 3×10^{-5} M concentration of cocaine. However, 1×10^{-4} M cocaine tended to increase the maximum contraction of the vas deferens to norepinephrine more than the 3×10^{-5} M concentration of cocaine. Further studies would be needed to determine if 1×10^{-4} M concentration of cocaine has a postsynaptic effect on the mouse vas deferens.

Cocaine pretreatment increased the maximum degree of contraction of the vas deferens to compounds which have an affinity for intraneuronal uptake (norepinephrine and phenylephrine) but had no significant effect on the maximum response to methoxamine. This suggests that the ability of cocaine to inhibit intraneuronal uptake may be related to the ability of cocaine to increase the maximum response to the adrenergic agonists. However, increases in the maximum degree of contraction are not generally attributed to alterations in the concentration of drug in the region of the receptor. They are attributed to a change in the receptor itself, an increase in the total number of receptors available, or an alteration in the tissue at a site other than the receptor (see Introduction). Which of these mechanisms was involved in temperature-dependent and cocaine-induced supersensitivity was not elucidated by the results from the present experiments. However, the data suggest

several possible explanations.

A possible explanation for the action of cocaine would be that it increases the number of receptors available to (-)-norepinephrine. Recently Kalsner (1972) demonstrated differential activation of the inner and outer muscle layers of the rabbit ear artery. The data indicated that exogenous (-)-norepinephrine, (±)-methoxamine, or K^+ was not able to diffuse to all layers of muscle before the response was terminated. In the mouse vas deferens the density of innervation is unusually high with neurons in close proximity to each muscle cell (see Introduction). For this reason, it is quite possible that as an agonist diffuses through the tissue it is quickly accumulated by the neurons and unable to diffuse completely through the muscle layers to all of the receptors. When the tissue is incubated with cocaine prior to the addition of an adrenergic agonist, intraneuronal uptake is inhibited. Therefore, when the agonist comes in contact with the tissue, it is capable of diffusing through more muscle layers, reacting with more receptors and activating more smooth muscle cells at one time.

Since the present data suggest that decreasing the bath temperature inhibits intraneuronal uptake, it is possible that reducing the bath temperature increases the ability of the adrenergic agonists to diffuse through the tissue. However, the increase in the

maximum response to methoxamine cannot be related to a decrease in neuronal uptake, since the concentration of methoxamine available for reacting with receptors is not affected by this means of amine inactivation. There are several possible explanations for the increased maximum degree of contraction of the mouse vas deferens to methoxamine.

One possibility is that decreasing the bath temperature inhibits extraneuronal uptake, thereby increasing the ability of methoxamine to diffuse to more receptors. Methoxamine does have some affinity for extraneuronal uptake in the rat heart (Iversen, 1967). In addition, Gillespie et al. (1970) demonstrated that lowering the bath temperature reduced the extraneuronal uptake of norepinephrine in cat spleen tissue slices. The effect of temperature on the extraneuronal uptake of the cat spleen was evident at temperatures of 20°C or lower. However, the ability of methoxamine to be retained extraneuronally in the mouse vas deferens has not been measured. In addition, the effect of temperature changes on extraneuronal uptake in the mouse vas deferens has not been determined.

A second possible explanation for the increase in maximum contraction to adrenergic agonists at 20°C is a transformation of beta to alpha receptors as the temperature decreases. This type of transformation has

been reported in the frog and rat heart (Kunos and Szentivanyi, 1968; Buckley and Jordan, 1970). Since beta adrenergic receptors are inhibitory in the vas deferens (Large, 1965; Takagi and Takayanagi, 1965; Ganguly and Bhattacharya, 1969), a decrease in their inhibitory activity would produce an increase in degree of contraction. One would expect this to be pronounced for agonists that activate both alpha and beta adrenergic receptors. However, the maximum response to methoxamine, which possesses beta receptor antagonist activity, was increased. Therefore, it is unlikely that a transformation of beta adrenergic receptors to alpha adrenergic receptors is responsible for the increase in the maximum response.

A third possible explanation is that decreasing the bath temperature increases the availability of extracellular Ca^{++} and/or produces a conformational change in the receptor. It cannot be determined from the present experiments if these mechanisms are responsible for the increased maximum response.

The fourth possible explanation for the increase in the maximum response of the vas deferens to methoxamine at 20°C is that reducing the temperature prolonged the response to these adrenergic agonists. Such a prolongation was observed; however, it was not quantitated. By prolonging the response, more receptors could be activated before the contractile mechanisms began to

return to the relaxed state.

In summary, it can be concluded that the increase in maximum degree of contraction to norepinephrine induced by decreased temperature or by cocaine pretreatment is related to inhibition of intraneuronal uptake by these treatments. An additional effect of temperature on extraneuronal uptake or on postsynaptic mechanisms involved in tissue response is suggested but not proven.

CONCLUSION AND SUMMARY

The effect of alteration of bath temperature on the isotonic contractions of the isolated mouse vas deferens has been measured. Decreasing the bath temperature from 37°C to 20°C increased the potency of and the maximum degree of contraction to (-)-norepinephrine. Pargyline or tropolone pretreatment did not prevent the effect of temperature on the response of the vas deferens to (-)-norepinephrine. At 37°C cocaine pretreatment also increased the potency of, and the maximum contraction to (-)-norepinephrine. In addition, cocaine pretreatment and decreased bath temperature had similar effects on the responses of the vas deferens to (-)-alpha-methyl norepinephrine, (-)-metaraminol, and (-)-phenylephrine and on the retention of ^{14}C-norepinephrine. However, although neither alteration of bath temperature or cocaine pretreatment had a statistically significant effect on the potency of methoxamine on the mouse vas deferens, the effects of these treatments on the maximum degree of contraction to methoxamine differed. Cocaine pretreatment had no effect on the maximum degree of contraction of the mouse vas deferens to methoxamine. In contrast, reducing the bath temperature from 37°C to 20°C produced a marked increase in the maximum contraction of the vas deferens to methoxamine.

The similarity of the effects of cocaine and decreased bath temperature on the potency of adrenergic agonists measured in the mouse vas deferens suggests that decreasing the bath temperature increases the potency of norepinephrine by inhibiting neuronal uptake. In addition, since the effect of temperature on the responses to norepinephrine are not prevented by pargyline or tropolone pretreatment, any possible change in the activity of MAO or COMT does not appear to be involved in temperature-dependent supersensitivity of the mouse vas deferens to (-)-norepinephrine. This lack of influence of MAO or COMT is further supported by the increased potency of (-)-phenylephrine and (-)-alpha-methyl norepinephrine at 20°C.

Cocaine pretreatment increased the maximum contraction of the vas deferens to (-)-norepinephrine and (-)-phenylephrine but not to methoxamine which suggests that the effect of cocaine on the maximum response may be related to the effect of cocaine on intraneuronal uptake. It is suggested that the increase in the maximum contraction after cocaine pretreatment may be a result of an increased ability for adrenergic agonists to diffuse to more smooth muscle cells and therefore, more receptors.

Since the data suggest that bath temperature reduction from 37°C to 20°C inhibits intraneuronal uptake, it is possible that reducing the bath temperature

increases the ability of the adrenergic agonists to diffuse to more receptors. However, the increase in maximum degree of contraction to methoxamine suggests that temperature has an additional effect either on extraneuronal uptake and/or on postsynaptic mechanisms involved in tissue response.

APPENDIX

TABLE 12

Effect of consecutive administration of adrenergic amines on the sensitivity of the mouse vas deferens

Agonist	°C[a]	-log Molar ED_{50} with S.E.M.			Maximum contraction (cm)[b] with S.E.M.			n[c]
		Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3	
(-)-Norepinephrine	37°C	5.36±.15	5.38±.07	5.48±.11	6.46±1.11	5.75±1.18	5.65±1.14	7
(-)-Norepinephrine	20°C	5.98±.15	5.76±.07	5.61±.06	5.83±.86	5.35±.84	5.22±.89	7
(±)-Norepinephrine	37°C	5.26±.09	5.06±.05	4.98±.08	7.22±.61	7.62±.60	7.71±.59	8
(±)-Norepinephrine	20°C	5.66±.13	5.43±.14	5.41±.16	6.95±.54	6.88±.62	6.76±.55	8
(-)-Phenylephrine	37°C	5.25±.09	5.37±.13	5.21±.09	3.20±.59	2.88±.64	2.53±.53	8
(-)-Phenylephrine	20°C	5.62±.10	5.40±.06	5.55±.21	3.78±.49	2.48±.43	2.44±.45	8
(-)-Norepinephrine[d]	37°C	5.42±.09	5.56±.16	5.84±.11[e]	5.33±.60	4.75±.64	4.40±.74[e]	8
(-)-Norepinephrine[d]	20°C	6.20±.16	6.09±.15	6.10±.14[e]	7.62±.70	5.87±.62	4.35±.66[e]	8
(±)-α-Methyl norepinephrine	37°C	4.69±.07	4.76±.10	4.65±.09	5.37±.96	4.32±.86	3.74±1.01	8
(±)-α-Methyl norepinephrine	20°C	4.64±.10	4.59±.11	4.60±.10	5.37±.48	3.52±.43	2.51±.39	8
(±)-Metaraminol	37°C	4.64±.07	4.64±.05	4.55±.08	7.55±.46	6.48±.57	5.56±.69	8
(±)-Metaraminol	20°C	4.54±.06	4.44±.04	4.36±.08	6.12±.58	5.20±.56	4.23±.60	8
(-)-Metaraminol	37°C	4.76±.07	4.77±.15	—	6.30±.35	5.11±.56	—	8
(-)-Metaraminol	20°C	4.82±.10	4.69±.08	—	5.90±.45	4.55±.40	—	8

[a]Bath temperature.

[b]Centimeters of pen deflection.

[c]Number of observations.

[d]Pretreated with pargyline 100 mg/kg 24 hrs prior to sacrifice.

[e]Obtained in the presence of cocaine 3×10^{-5} M.

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