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EFFECTS OF PROTEIN DENATURANTS ON THE ADRENERGIC CONTRACTILE MECHANISM OF ISOLATED SEMINAL VESICLES OF MICE

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Abstract—Exposure of mouse seminal vesicles to various protein denaturants altered responses to norepinephrine and KCl. These denaturants can be classified into two groups from the results of the experiments: (1) urea (1 M, 10 and 40 min) and guanidine hydrochloride (0·1 M, 10 min) depressed norepinephrine-induced contractions selectively; and (2) *N,N*-dimethylformamide (1·3 M, 15 min), *N,N*-dimethylacetamide (1·3 M, 10 and 20 min) and dioxane (0·6 M, 10 min) reduced contractions induced by KCl in preference to those induced by norepinephrine. These findings interestingly agreed with the classification of denaturants based on the conformation of products formed by protein denaturation *in vitro*. Antagonism of ephedrine (4×10^{-4} M) against norepinephrine was of the competitive type under normal conditions, but exposure to *N,N*-dimethylacetamide (1·3 M, 10 and 20 min) altered the antagonism to the noncompetitive type. The blocking action of Dibenamine was not affected by any of these denaturants. Possible sites of denaturation are discussed.

DRUG receptors are assumed to contain protein as essential components in their structures, and their biological activities are altered by heat, change in pH, enzyme or urea.[1] It is generally accepted that secondary and tertiary structures of proteins are responsible for their biological activities. The term "protein denaturation" sounds like a nonspecific phenomenon, but various denaturants show their own characteristics in interaction with proteins.[2] Guanidine hydrochloride, urea, acid pH or organic acids (e.g. dichloroacetic acid) tend to destroy the α-helical, ordered structure of native protein and lead to the formation of denatured products that are randomly coiled, or that consist partly of randomly coiled regions and partly of globular regions. The action of 2-chloroethanol, dioxane and *N*-methyl-substituted amides (e.g. dimethylformamide) falls into a different category; these agents tend to form an α-helical conformation.

Recently, protein denaturants have been used in studies on the drug receptor mechanism.[3–6] In the present experiment, the author used six denaturants, two of which were selected from the first group and four from the second. Differences in the effects of the denaturants on norepinephrine-induced and KCl-induced contractions in isolated mouse seminal vesicles were observed. Further, the influence of the denaturants on ephedrine–norepinephrine antagonism and on the α-adrenergic blocking action of Dibenamine were observed.

METHODS

Male dd-strain mice weighing between 20 and 25 g were used. The bilateral seminal vesicles were removed and pressed gently with tweezers to exhaust secreta. Each

unilateral vesicle was mounted separately in a 50-ml organ bath. One served for the control and the other for the denaturation experiments. The bathing fluid was Tyrode's solution (containing in grams per liter: NaCl, 8·0; KCl, 0·2; $CaCl_2$, 0·2; $MgCl_2$, 0·1; NaH_2PO_4, 0·05; $NaHCO_3$, 1·0; glucose, 1·0) aerated with 95% O_2 and 5% CO_2 and maintained at 36 ± 0·5°. Contractile responses of the preparations were recorded isotonically on smoked paper.

Denaturants. Guanidine hydrochloride and urea were selected as denaturants which tend to destroy the α-helical, ordered structure of native protein and lead to the formation of products that are randomly coiled.

Dioxane, 2-chloroethanol, *N,N*-dimethylformamide and *N,N*-dimethylacetamide were selected as denaturants which tend to form an α-helical conformation.

Dose–response curves of norepinephrine and KCl. A schema of the set of kymographs obtained is shown in Fig. 1, A–F. The control (A–C) and experimental tissues (D–F) were given parallel treatment so that each tissue could serve as its own control. Thirty

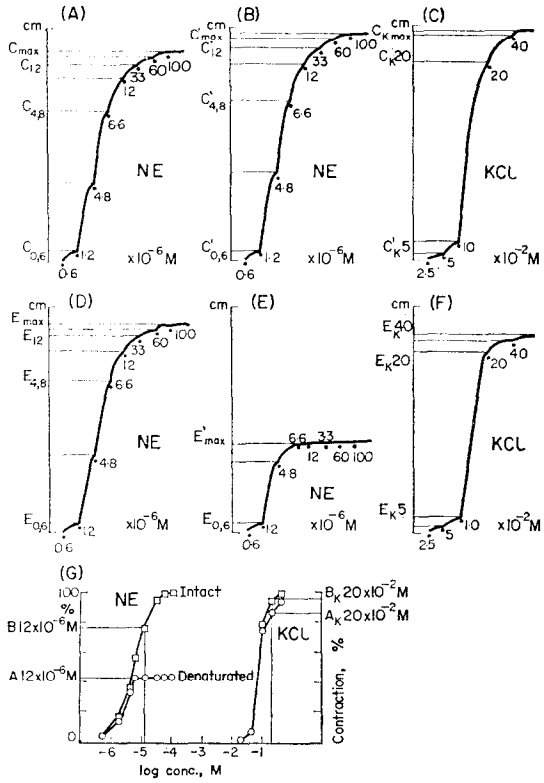

FIG. 1. (A–F) Schema of kymograph of contractions induced by norepinephrine (NE) and KCl in control (A–C) and experimental (D–F) seminal vesicles of the mouse. Norepinephrine or KCl was added cumulatively. Values by the curves indicate the final concentrations. The details are described in the text. (G) Calculated dose–response curves of norepinephrine (NE) and KCl in intact and denatured seminal vesicles. The details are described in the text.

min after setting up the isolated seminal vesicles in the organ bath, cumulative dose–response curves to norepinephrine were determined (Fig. 1, A and D). The vesicles were washed with Tyrode's solution and allowed 15 min for recovery. The experimental preparations were exposed to various denaturants for varying periods of time by exchanging the bathing fluid for Tyrode's solution containing various concentrations of denaturants maintained at 36 $\pm$ 0·5°. Then the vesicles were washed six times with 50 ml of Tyrode's solution. One hr after the last washing, the second dose–response curves to norepinephrine were obtained (Fig. 1, B and E). Fifteen min after the vesicles were washed, cumulative dose–response curves to KCl were also obtained, in order to estimate the specificity of influence of a denaturant to norepinephrine-induced contraction (Fig. 1, C and F).

Calculation for correcting responses of experimental preparations not yet exposed to a denaturant. Two vesicles from one mouse did not show identical amplitude of contraction and the responses of seminal vesicles to norepinephrine developed gradually over 2·5–3·5 hr (as shown in Fig. 1, A and B). In order to compare the response of the denatured vesicles (Fig. 1E) with the response of the same vesicle not yet denatured (Fig. 1D), the responses of the experimental vesicles not yet denatured were corrected. Figure 1G shows the dose–response curves calculated from kymographs showed in Fig. 1, A–F.

$$Bx = \frac{Ex \times \dfrac{C'x}{Cx}}{E_{max} \times \dfrac{C'_{max}}{C_{max}}} \times 100 \qquad (1)$$

$$Ax = \frac{E'x}{E_{max} \times \dfrac{C'_{max}}{C_{max}}} \times 100 \qquad (2)$$

Bx = the calculated per cent contraction induced by dose x of norepinephrine in the experimental preparation not yet denatured; Ax = the calculated per cent contraction induced by dose x of norepinephrine in the denatured experimental preparation; Cx = the response to dose x of norepinephrine estimated from the first curve of the control preparation (Fig. 1A); C_{max} = the maximal response to norepinephrine estimated from the first curve of the control preparation (Fig. 1A); Ex = the response to dose x of norepinephrine estimated from the first curve of the experimental preparation (Fig. 1D); E_{max} = the maximal response of norepinephrine estimated from the first curve of the experimental preparation (Fig. 1D); $C'x$ = the response to dose x of norepinephrine estimated from the second curve of the control preparation (Fig. 1B); C'_{max} = the maximal response to norepinephrine estimated from the second curve of the control preparation (Fig. 1B); $E'x$ = the response to dose x of norepinephrine estimated from the second curve of the experimental preparation (Fig. 1E).

For comparison of the response to KCl of the control and the experimental preparation, the response to KCl was expressed as the ratio of the maximal response to nore-

pinephrine. The values of per cent contraction of KCl (Fig. 1G) were calculated as follows:

$$B_Kx = \frac{\dfrac{C_Kx}{C_{max}}}{\dfrac{C_{Kmax}}{C_{max}}} \times 100 = \frac{C_Kx}{C_{Kmax}} \times 100 \quad (3)$$

$$A_Kx = \frac{\dfrac{E_Kx}{E_{max}}}{\dfrac{C_{Kmax}}{C_{max}}} \times 100 = \frac{E_Kx \times C_{max}}{C_{Kmax} \times E_{max}} \times 100 \quad (4)$$

B_Kx = the per cent contraction induced by dose x of KCl in the control preparation; A_Kx = the per cent contraction induced by dose x of KCl in the denatured experimental preparation; C_Kx = the maximal response to KCl estimated from the control preparation; E_Kx = the response to dose x of KCl estimated from the denatured experimental preparation.

Use of antagonists. In the experiments using antagonists, the control and experimental vesicles were obtained by the method mentioned above and were given parallel treatment. Thirty min after setting up the isolated seminal vesicles in the organ bath, experimental vesicles were exposed to various denaturants for varying periods of time by exchanging the bathing fluid for Tyrode's solution containing various concentrations of denaturant maintained at 36 $\pm$ 0·5°. Then the vesicles were washed six times with 50 ml of Tyrode's solution. One hr after the last washing, cumulative dose–response curves to norepinephrine were obtained. Twenty min later, the vesicles were washed three times in the presence of ephedrine (4×10^{-4} M) or Dibenamine (10^{-6} M), and the second dose–response curves to norepinephrine were obtained. Ephedrine or Dibenamine was allowed to act for 3 min before addition of norepinephrine.

RESULTS

Effects of denaturants on responses to norepinephrine and KCl. Figure 2 shows the effects of guanidine hydrochloride and urea at varying concentrations and times. Decreases in the slope and maxima of the dose–response curves for norepinephrine were obtained for the seminal vesicles treated with these denaturants. A 10-min exposure to 0·1 M guanidine hydrochloride reduced the amplitude of the maximal response to norepinephrine by 55 per cent, but that to KCl was only reduced by 14 per cent. The maximal responses to norepinephrine of seminal vesicles treated with 1 M urea were 87 per cent (10-min exposure) and 60 per cent (40-min exposure) for the intact preparation, whereas those to KCl were 100 and 78 per cent. With 2 M urea (10 and 20 min), the responses to both stimulants were reduced to much the same degree. In this case it may be accepted that damage by the denaturant reached the contractile apparatus or a stage of the excitation–contraction coupling which is common to both norepinephrine and KCl.

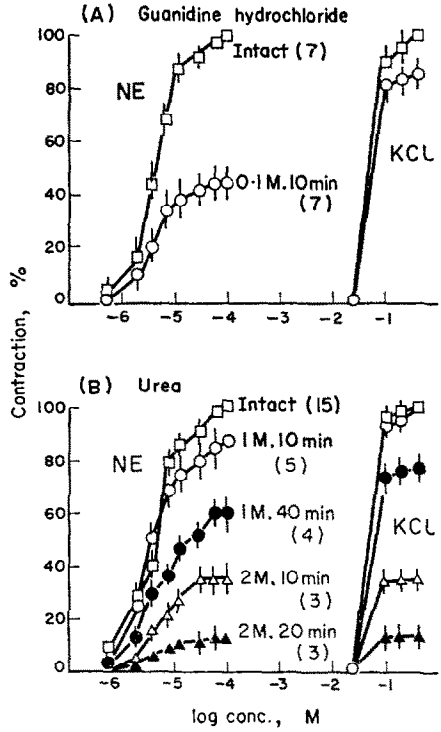

FIG. 2. Effects of guanidine hydrochloride (A) and urea (B) on responses of seminal vesicles to norepinephrine (NE) and KCl. Values in parentheses represent numbers of experiments; vertical bars indicate standard errors of means.

Pretreatment with *N,N*-dimethylformamide (1·3 M, 15 min) or *N,N*-dimethylacetamide (1·3 M, 10 and 20 min) decreased the maximal responses to KCl more than those to norepinephrine, in contrast to the effects of 1 M urea or 0·1 M guanidine hydrochloride (Fig. 3). A 10-min exposure to 0·6 M dioxane reduced the maximal response to KCl more than that to norepinephrine (Fig. 4B).

2-Chloroethanol (0·2 M, 10 and 15 min) depressed contractions induced by norepinephrine and KCl to much the same degree (Fig. 4A). Equipotent inhibition of norepinephrine-induced and KCl-induced contractions was also observed in the vesicles which were exposed to 2 M urea for 10 and 20 min, but the degree of inhibition was much greater than that caused by 2-chloroethanol.

None of the denaturants employed induced parallel shifts of the dose–response curves for norepinephrine, which indicates the noncompetitive inhibition of these denaturants.

Effects of denaturants on ephedrine–norepinephrine antagonism and on the α-adrenergic blocking action of Dibenamine. Among the differences observed in the effects of the six denaturants were the irreversible blockade produced by Dibenamine and the competitive antagonism produced by ephedrine. Dibenamine (10^{-6} M) inhibited completely the response to norepinephrine of all the denatured preparations as well as the controls. Ephedrine–norepinephrine antagonism in the control preparations

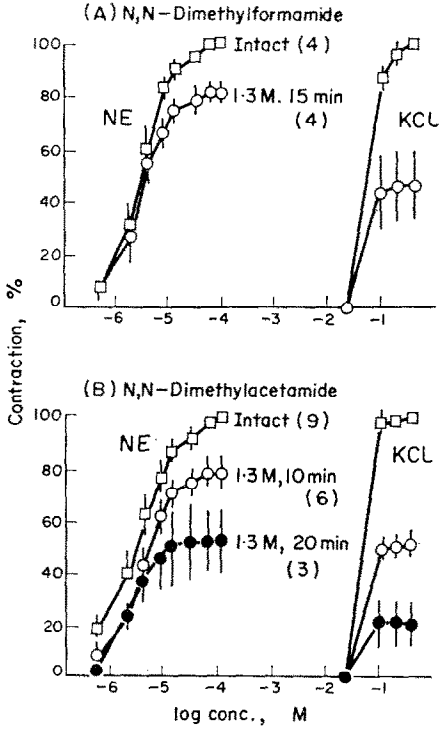

FIG. 3. Effects of *N,N*-dimethylformamide (A) and *N,N*-dimethylacetamide (B) on responses of seminal vesicles to norepinephrine (NE) and KCl. Values in parentheses represent numbers of experiments; vertical bars indicate standard errors of means.

was competitive. When the seminal vesicles were exposed to 1·3 M *N,N*-dimethylacetamide for 10 or 20 min, the dose–response curves for norepinephrine in the presence of ephedrine (4×10^{-4} M) declined without a parallel shift. Other denaturants tested had no influence on the competitive antagonism of ephedrine (Fig. 5).

DISCUSSION

The following characteristics of mouse seminal vesicles in relation to sympathomimetic amines have been demonstrated by previous experiments:[7,8] (1) The adrenergic receptors belong to the alpha type. (2) The vesicles respond to only several sympathomimetic amines, e.g. norepinephrine, epinephrine, phenylephrine and dopamine under normal conditions. In the presence of $BaCl_2$ ($2{\cdot}8 \times 10^{-3}$ M), isoproterenol and indirectly acting amines also produce contraction, and α-blocking agents diminish the contraction. (3) The response to epinephrine is competitively inhibited by many sympathomimetic amines, 5-hydroxytryptamine, histamine and high concentrations of β-blocking agents.

It is now generally supposed that two distinct calcium pools are available and that potassium utilizes calcium from a more labile, accessible pool than does norepinephrine.[9–12]

The adrenergic contractile mechanism is injured by protein denaturants. The sites of damage may be one or more of the stages of excitation–contraction coupling, e.g.

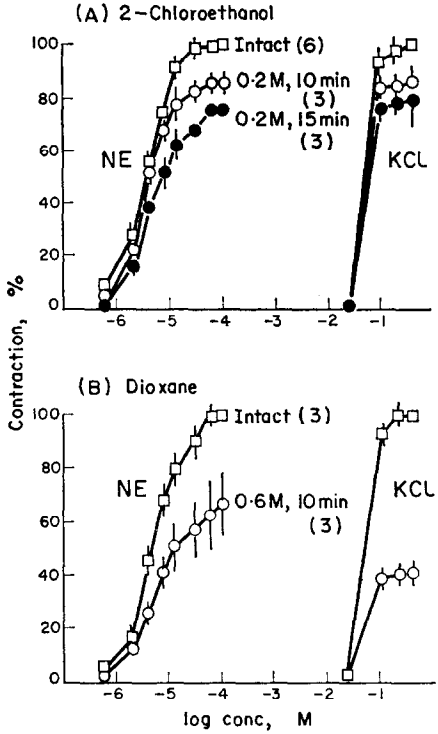

FIG. 4. Effects of 2-chloroethanol (A) and dioxane (B) on responses of seminal vesicles to norepinephrine (NE) and KCl. Values in parentheses represent numbers of experiments; vertical bars indicate standard errors of means.

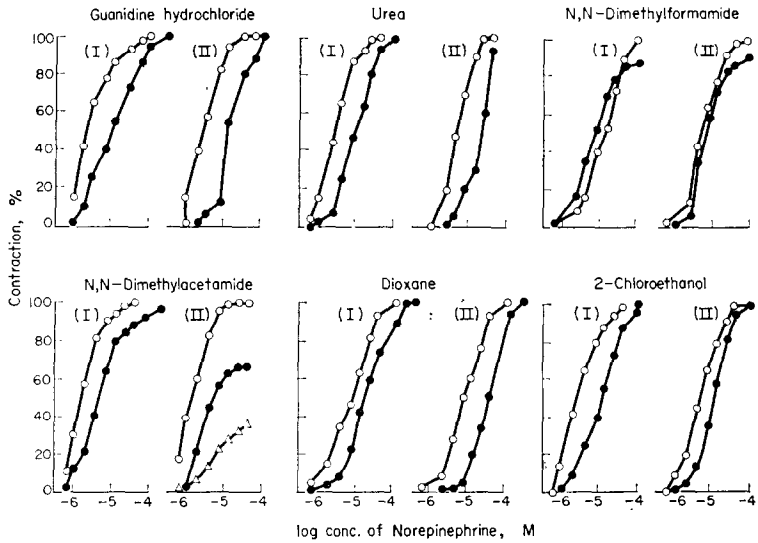

FIG. 5. Effects of denaturants on antagonism of ephedrine (4×10^{-4} M) against norepinephrine. The number of experiments was four or five for each denaturant. (I) control; (II) preparation exposed to a denaturant without ephedrine (○——○), or in the presence of ephedrine (4×10^{-4} M) (●——●), (△——△). Conditions of denaturation were as follows: guanidine hydrochloride (0·1 M, 10 min), urea (1 M, 40 min), *N,N*-dimethylformamide (1·3 M, 15 min), *N,N*-dimethylacetamide [1·3 M, 10 min (●——●) and 20 min (△——△)], dioxane (0·6 M, 10 min) and 2-chloroethanol (0·2 M, 10 min).

the adrenotropic receptor, the norepinephrine-specific calcium pool or the contractile apparatus. Selective inhibition of norepinephrine-induced contraction by guanidine hydrochloride (0·1 M, 10 min) and urea (1 M, 10 and 40 min) may be interpretable on the assumption that these denaturants specifically affected the adrenotropic receptors or the calcium pool, or both, independently of that utilized by KCl. The preparations treated with these two denaturants maintained significantly higher maximal responses to KCl than to norepinephrine, suggesting that the selective depression of norepinephrine-induced contraction was not related to damage of the conductive membrane or contractile elements. Kubota *et al.*[6] reported a similar selective blockade of norepinephrine in the guinea pig and rat vas deferens treated with urea.

In contrast to the case mentioned, exposure of the seminal vesicles to *N,N*-dimethylformamide (1·3 M, 15 min), *N,N*-dimethylacetamide (1·3 M, 10 and 20 min) or dioxane (0·6 M, 10 min) caused greater depression of KCl-induced contraction than of norepinephrine-induced contraction. Specific inhibition of KCl-induced contraction by these three denaturants suggests that damage occurred in a part of the KCl-specific pathway of excitation–contraction coupling. Edman and Schild[13, 14] suggested that epinephrine can produce contractile responses which are not mediated by depolarization, but which probably involve mobilization of bound calcium.

If norepinephrine had the same mechanism as epinephrine, damage to the conducting membrane would not reduce contraction induced by norepinephrine as greatly as it decreased KCl-induced contraction.

From the above results, denaturants may be classified into two groups: (1) those which depress norepinephrine-induced contraction selectively, and (2) those which preferably reduce KCl-induced contraction. Urea and guanidine hydrochloride fall into the first category, and dioxane, *N,N*-dimethylformamide and *N,N*-dimethylacetamide fall into the second. These results interestingly agreed with classification of denaturants based on the conformation of products formed by protein denaturation. Urea and guanidine hydrochloride lead to the formation of products that are randomly coiled or that consist partly of randomly coiled regions and partly of globular regions. On the other hand, protein denaturation by dioxane, *N,N*-dimethylformamide or *N,N*-dimethylacetamide does not tend to destroy the α-helical regions of the protein molecule.

Kalsner *et al.*[12] found that exposure of rabbit aortic strips to SKF 525A ($2{\cdot}6 \times 10^{-5}$ M) for 30 min blocked KCl-induced contractions without reducing the response to moderate concentrations of norepinephrine or phenylephrine. Contractions induced by calcium in aortic strips incubated in a calcium-free, high-potassium solution were almost completely prevented by a 15-min exposure to SKF 525A. Responses induced by calcium added in the presence of norepinephrine in a calcium-free medium were significantly, but only partially, depressed by a 15-min exposure to SKF 525A. Kalsner *et al.*[12] concluded that SKF 525A selectively blocks some step in the process by which KCl-induced depolarization promotes the movement of extracellular or superficially bound calcium (or both) to contractile elements, but does not directly interfere with mobilization of calcium by norepinephrine from a separate, firmly bound source. Similar events can also be assumed to occur in seminal vesicles treated with *N,N*-dimethylformamide (1·3 M, 15 min), *N,N*-dimethylacetamide (1·3 M, 10 and 20 min) or dioxane (0·6 M, 10 min). One of the reasons for this assumption is the

fact that SKF 525A is a surfactant;[15] proteins denatured by surfactants maintain a large degree of ordered structure, although the detailed structure may often be quite different from that of the native protein. Therefore, as in the case of *N,N*-dimethylformamide, *N,N*-dimethylacetamide or dioxane, the mechanism by which SKF 525A selectively blocks KCl-induced contractions may be the denaturation of protein which is related to calcium mobilization.

Although 2-chloroethanol (0·2 M, 10 and 15 min) did not reduce as greatly as did 2 M urea (10 and 20 min), equipotent depression of both norepinephrine-induced and potassium-induced contractions was observed in the preparations exposed to 2-chloroethanol. It is supposed that since norepinephrine- and potassium-specific parts in excitation–contraction coupling were resistant to the action of 2-chloroethanol, the contractile element was damaged prior to the initial, specific parts. However, it is undeniable that norepinephrine- and potassium-specific parts were damaged to much the same degree by exposure to 0·2 M 2-chloroethanol for 10 or 15 min. This denaturant does not tend to destroy the α-helical region of the protein molecule. If more suitable concentrations and times are employed, some specific influences may be observed.

In the above discussion, the selective inhibition of norepinephrine- or KCl-induced contractions by denaturants was interpreted by the difference in the degree of structural damage at each specific step involved in the contraction induced by each stimulant. However, another explanation can be considered: Although both types of stimulants may be equal in the degree of denaturation of some structure which mediates the contractile response, norepinephrine- and KCl-induced contractions may be unequal by nature in the degree to which their physiological function depends on the conformational integrity of the structure. For example, it is likely that guanidine hydrochloride introduced an equal conformational change into both structures concerned with norepinephrine and KCl, but the efficiency of the excitation–contraction coupling of the denatured vesicle was reduced to 86 per cent with KCl and to 45 per cent with norepinephrine.

Denaturation of seminal vesicles induced decreases in the slopes and maxima of the dose–response curves of norepinephrine throughout the experiments. Possible reasons for this phenomenon are reduction in the amounts of active receptors and/or partial damage of the contractile mechanisms, including the calcium utilization step and contractile protein.

It is interesting that ephedrine antagonism against norepinephrine was altered from the competitive to the noncompetitive type by treatment with *N,N*-dimethylacetamide, whereas other denaturants did not affect the type of antagonism. Satisfactory explanation of this is difficult, but it is likely that in the *N,N*-dimethylacetamide-treated preparation, ephedrine bound to receptor irreversibly and resulted in a noncompetitive antagonism. A lipid[16] or nonlipid[17] substance (probably protein) has been proposed as the site to which irreversible α-adrenergic blocking agents bind in order to exert their physiological activities. Whichever it may be, protein seems to be required for the receptor to mediate physiological activities.

The term "protein denaturation" has been used in this study to explain selective inhibition. Tanford[2] defined protein denaturation as follows: "it is simply a major change from the original native structure without alteration of the amino acid sequence, i.e. without severance of any of the primary chemical bonds which join

one amino acid to another". However, the event which occurred in the seminal vesicles exposed to denaturants may be distinguished from this defined "protein denaturation"; it may not be a major conformational change of a whole protein molecule, but only a minor one. Concentrations of denaturants employed in this experiment were lower than those which can elicit sufficient denaturation in protein, e.g. lysozyme, *in vitro*.[2]

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