Use of Isolated Strips of Cat Spleen for the Assay of α-Adrenergic Blocking Compounds

By ROBERT K. BICKERTON, WILLIAM T. ROCKHOLD, and EDWIN R. MICALIZZI

The response of isolated strips of cat spleen to epinephrine, norepinephrine, and acetylcholine was studied. It was found that spleen strips suspended in glucose-deficient Tyrode's solution exhibited marked sensitivity to the catecholamines and were relatively insensitive to acetylcholine. This differential sensitivity was utilized to advantage in the development of an *in vitro* assay procedure for α-adrenergic blocking agents. In addition, the method offers a qualitative index to the degree of tissue receptor binding of the antagonists.

MANY *in vitro* preparations have been used for the assay of sympathetically active compounds. Brugger (1), Rothlin and Brugger (2), and Rothlin (3) used the isolated seminal vesicle of the guinea pig to assay for the adrenolytic effects of the ergot alkaloids. Stone and Loew (4) used this same preparation as an assay for antiadrenaline drugs and Leitch, Liebig, and Haley (5) used the rat's seminal vesicle for the assay of certain sympatholytic drugs. Burn, Finney, and Goodwin (6) described several *in vitro* techniques for the assay of adrenaline and noradrenaline. Many past reports, and more recently those of Scott (7), Burn and Rand (8, 9), Daly and Scott (10), and Brandon and Rand (11) have described studies concerning the response of the mammalian spleen, both *in situ* and *in vitro*, to a number of drugs. None of these reports has described the use of this organ for the assay of adrenergic blocking compounds. This report deals with the response of isolated strips of cat spleen to epinephrine, norepinephrine, and acetylcholine, and the use of this organ for the assay of adrenergic blocking agents.

EXPERIMENTAL

Methods.—In all experiments, cats weighing 2 to 3 Kg. and unselected as to age or sex were used. The animals were anesthetized by an intraperitoneal injection of 0.7 ml./Kg. of diallylbarbituric acid-urethane.[1] The spleen was exteriorized through a lateral abdominal incision and then removed by section of all vasculature at the junction of splenic pedicle and dorsal hilus. The spleen was then rinsed and placed in warmed Tyrode's solution containing one-half the usual amounts of sodium bicarbonate and anhydrous dextrose. The sides and ends of the spleen were trimmed to leave a central rectangular strip measuring approximately 9 × 1.5 cm. This strip was divided into two equal portions, each measuring 4.5 × 1.5 cm., and each half was placed into a separate 70-ml. muscle bath containing oxygenated (95% oxygen, 5% carbon dioxide) Tyrode's solution of the previously mentioned composition. The bath was maintained at 39°. Each spleen strip was connected to an isotonic ink-writing lever so that contractions could be recorded on a kymograph. A magnification ratio of 13:1 was used and the strips were placed under constant tension of 5.0 Gm. Acetylcholine chloride, *l*-epinephrine bitartrate, and *l*-norepinephrine bitartrate were dissolved in 0.9% sodium chloride just before use. Ergotamine tartrate and tolazoline hydrochloride were prepared by dilution of the commercially available ampuls with 0.9% sodium chloride prior to use. Dibenamine hydrochloride was prepared as a 5% stock solution in propylene glycol and diluted with 0.9% sodium chloride just prior to use. Guanethidine sulfate was dissolved in dilute hydrochloric acid solution and buffered to pH 6 with sodium bicarbonate. Final dilutions of guanethidine were made with 0.9% sodium chloride solution. Doses of all drugs were added to the baths in 0.35- to 2.1-ml. volumes except in the assay procedure where all doses of drugs were added in 1.0-ml. volumes.

In performance of the assay, *l*-epinephrine was added to each bath to give a concentration of 0.5 mcg./ml. and kept in contact with the spleen strip for 5 minutes. During this time maximal contraction to this dose occurred. The epinephrine was then drained and the strip washed and allowed to equilibrate for 10 minutes, and then the wash was repeated. If complete relaxation of the strips did not occur within this interval, the writing lever was manually returned to base line. Five minutes after the last wash, *l*-epinephrine was again added and the above procedure repeated. Epinephrine was added at 20-minute intervals until four consecutive responses were obtained which did not vary more than 5% from their mean. Immediately after the second wash from the last control response, the antagonists were added to the baths in varying concentrations and allowed to remain in contact with the strip for 5 minutes. Without further washing, *l*-epinephrine was again added to the bath and the contraction recorded. The strip was washed as described and a minimum of two more responses to epinephrine were obtained at the usual 20-minute intervals in order to determine how readily the antagonists were removed from their receptor sites. Only one dose of an antagonist was administered to any one spleen strip, with the exception of tolazoline which was administered in repeated doses to

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[1] Dial-Urethane (Ciba)

three strips. Per cent inhibition of epinephrine response from a given dose of antagonist was calculated from the difference in contraction heights of the mean of the four consecutive responses prior to the antagonist, and the response to epinephrine immediately following the antagonist. ED_{50} values for the antagonists were determined with the aid of an IBM 1620 digital computer programmed to determine the log-dose, probit-inhibition least squares regression line, its slope, and 95% confidence limits for the slope for each antagonist. All values were obtained at printout.[2]

The effect of time of contact of the antagonist with the spleen strip was studied in two strips from the same animal. Tolazoline and dibenamine were selected as the antagonists. The standard assay procedure was carried out excepting that the antagonists were allowed to remain in contact with the spleen for only 5 minutes in one strip and for 10 minutes in the remaining strip.

RESULTS

Effect of Epinephrine, Norepinephrine, and Acetylcholine.—Addition of *l*-epinephrine or *l*-norepinephrine to the spleen strips in varying concentrations produced contractions which were initially rapid, and then followed by a slow component which reached a maximum within 5 minutes. The response of the spleen strips to a given dose of these catecholamines increased in magnitude over the first four or five responses and then remained relatively uniform over the next several hours. Dose-response curves obtained from uniformly reacting strips were found to be logarithmically linear throughout a concentration range of 0.05 to 1.0 mcg. free base/ml. Some variation in reactivity to these catecholamines was found with different strips, although all spleen strips tested were more sensitive to epinephrine than to norepinephrine (see Fig. 1).

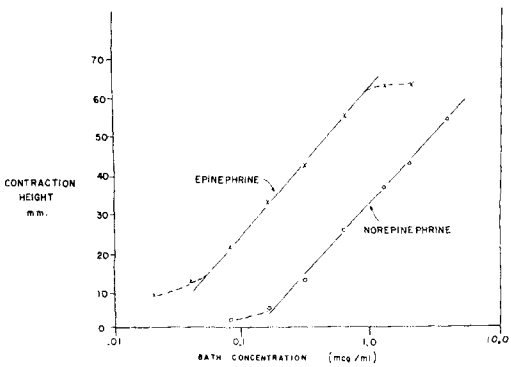

Fig. 1.—Log dose response curves for epinephrine and norepinephrine obtained from uniformly reacting cat spleen strip.

The contractile response of isolated cat spleen strips to acetylcholine, when present, was found to be moderate throughout a concentration range of 10 to 430 mcg./ml. When administered at 5-minute intervals, tachyphylaxis to acetylcholine rapidly developed, often after a single dose, and almost always within four repeated doses, although longer intervals between doses delayed the onset of tachyphylaxis (see Fig. 2).

Assay of Adrenergic Blocking Agents.—Table I lists the ED_{50} values for the α-adrenergic blocking agents dibenamine, ergotamine, and tolazoline. Since the slopes of the log-dose inhibition curves were not parallel,[3] potency comparisons were based

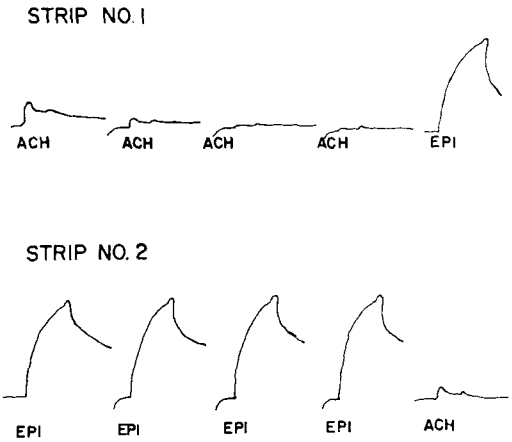

Fig. 2.—Development of tachyphylaxis to acetylcholine while the responses of spleen strip to epinephrine are unimpaired. EPI, 35 mcg. *l*-epinephrine; ACH, 700 mcg. acetylcholine chloride.

upon the ED_{50} values. With respect to the ED_{50} values, the following relationship, in order of decreasing potency, was found: ergotamine > dibenamine > tolazoline. Guanethidine, a sympatholytic agent, in bath concentrations ranging from 10 to 200 mcg./ml., did not inhibit, but in some trials enhanced epinephrine-induced contractions of isolated strips of cat spleen. Guanethidine alone exerted intrinsic contractile activity on the spleen strips in concentrations of 50 to 200 mcg./ml.

Degree of Tissue Receptor Binding of the Antagonists.—Since this assay procedure used repetitive washing and stimulation of the spleen strip by epinephrine, the method offers an index to the degree of binding of the antagonists at tissue receptors. Figure 3 shows the effects of the antagonists when added to the bath at their approximate ED_{50} concentration and the effects of repeated washing and stimulation by epinephrine. It can be seen that dibenamine produced progressive inhibiton of epinephrine-induced contraction seven though the solution containing the antagonist had been drained from the bath. Maximum inhibition of epinephrine by a given dose of ergotamine occurred within 5 minutes and was relatively prolonged. Tolazoline produced an immediate (within 5 minutes) inhibition of epinephrine but was more readily removed from binding sites by fewer washing cycles. The degree of receptor binding of the antagonists occurred in the following order of decreasing affinity for tissue receptors: dibenamine > ergotamine > tolazoline.

In determining the effects of antagonist contact time on the inhibition of epinephrine, it was found

[2] The authors wish to express their sincere appreciation to Dr. R. L. Beinert and G. V. O'Bleness for the statistical analysis used in this study.

[3] Slope parallelism was tested by the method of Davies (12).

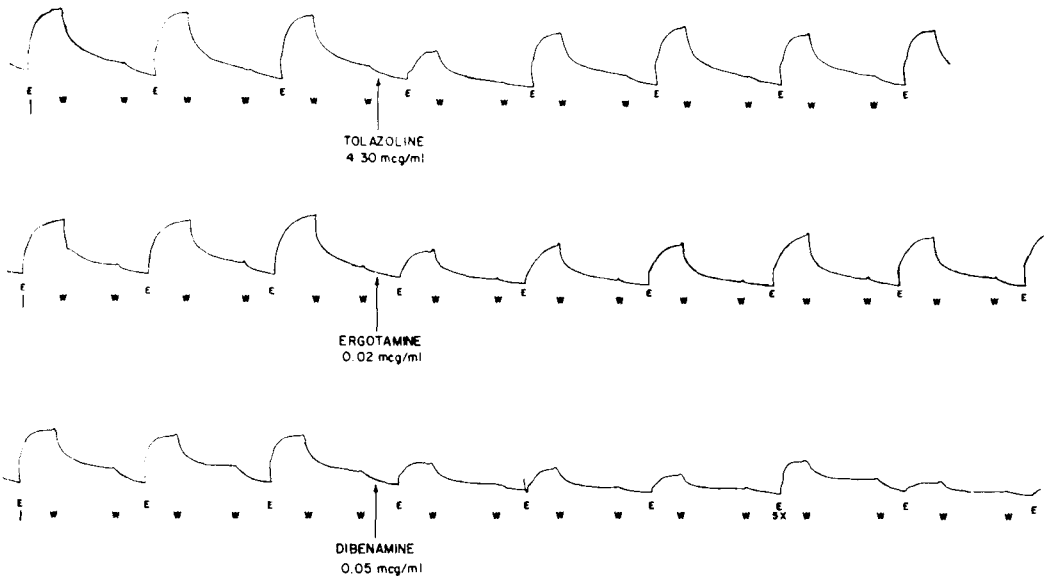

Fig. 3.—The effects of the antagonists when administered at their approximate ED_{50} concentrations on contractile responses of is olatedcat spleen to epinephrine (see text). E, Addition of 35 mcg. of epinephrine; W, wash.

that an increase in the time during which tolazoline was in contact with the spleen did not increase the inhibition of epinephrine by this antagonist but rather a decrease was observed. However, dibenamine produced greater inhibition after 10 minutes contact time than after 5 minutes contact (see Table II).

General Observations.—Throughout this study it has been our experience that the use of isolated strips of cat spleen for the assay of adrenergic blocking agents offers a major advantage over previous *in vitro* techniques in the relative durability of this organ. No special care in handling or removal from the animal is required. The organ is easily accessible through a small lateral incision which may be sutured after removal of the spleen. We have refrigerated excised spleen in Tyrode's solution containing a small amount of blood and have found that refrigeration for as long as 48 hours only slightly diminished the sensitivity to epinephrine. Longer periods of time resulted in greater decrease in sensitivity.

DISCUSSION

The contractile response of isolated cat spleen strips to *l*-epinephrine and *l*-norepinephrine was found to be logarithmically linear over a concentration range of 0.05 to 1.0 and 0.2 to 5.0 mcg. free base/ml., respectively. Responses to these catecholamines were reproducible over a period of many hours while those to administered acetylcholine under the conditions imposed by the assay were not. The order of potency of the three test antagonists was not in complete agreement with the data presented by Nickerson (13) who states that in potency, the ergot alkaloids occupy, in most instances, an intermediate position between the β-haloalkylamines and the imidazoline derivatives. The higher relative potency of ergotamine in our preparation could be explained on the basis of temporal relationships since it is known that the onset of dibenamine blockade is not immediate and that the degree of adrenergic blockade progresses with time up to the point of maximum effect. In our preparation, the dibenamine was in contact with the spleen strips for only 5 minutes. It is conceivable, therefore, that maximum blockade did not occur during this time. Further support of this suggestion was obtained from the fact that progressive inhibition did occur with dibenamine-treated strips, even though the bath containing the dibenamine had been drained and replaced with fresh Tyrode's solution. On the other hand, blockade produced by ergotamine was immediate and the degree of blockade was, therefore, accurately determined after 5 minutes contact time. The high potency of dibenamine and ergotamine in comparison to tolazoline is in agreement with Nickerson (13).

The finding that guanethidine did not inhibit epinephrine is in agreement with the data of Maxwell, *et al.* (14), who demonstrated the purely sympatholytic (as opposed to adrenolytic) action of this agent.

In considering the degree of tissue receptor binding of the antagonists, our results are in agreement with the data described by others (13). The progressive inhibition produced by dibenamine is similar to that which occurs in the intact animal and has been explained on the basis of the transformation from the equilibrium to the nonequilibrium type of blockade, presumably through the formation of ethylene immonium and/or vinyl intermediates, which then react chemically with cell constituents to produce a prolonged blockade. In this same regard, Axelrod *et al.* (15), reported that dibenamine did not disappear rapidly from the body of dogs but was extensively localized in body fat. These authors also found a close correlation between the degree of adrenergic blockade and the concentration of dibenamine in fatty tissue.

The rapidity with which tolazoline can be removed

TABLE I.—RESULTS OF THE ASSAY

Drug	No. Strips	Dose Range, mcg./ml.	ED_{50}, mcg./ml. (95% Confidence Limits)	Slope	95% Confidence Limits
Dibenamine	14	0.01 –1.0	0.05 (0.04–0.06)	2.4	±0.8
Tolazoline	11	1.0 –24.0	4.30 (3.37–5.50)	1.8	±0.5
Ergotamine	18	0.001–0.8	0.02 (0.01–0.03)	0.8	±0.2
Guanethidine	2	1.0 –200.0	>200	0.0	...

TABLE II.—EFFECT OF CONTACT TIME ON EPINEPHRINE INHIBITION

	Contact time	
	5 min.	10 min.
Tolazoline	52.8[a]	46.9[a]
Dibenamine	37.6	47.4

[a] Per cent inhibition.

from tissue receptors suggests that the binding forces involved are small and that removal from binding sites is dependent upon the distribution coefficient of the substance between tissue receptor and bathing medium. Brodie, *et al.* (16), have shown that tolazoline is only 23% plasma protein bound in the intact dog. These authors also found a relatively low degree of tissue localization of this agent and a relatively short biological half-life.

We have found this preparation to be an excellent tool for the investigation of drug action at adrenergic receptors. The relative ease in handling this tissue offers a distinct advantage over other methods. Investigations of the mechanism of action of a number of drugs on this organ are in progress.

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Synthesis of Some N- and S-Substituted Derivatives of 2-Aminobenzenethiol

By CHARLES S. DAVIS, GLENN L. JENKINS, ADELBERT M. KNEVEL, and CHARLES PAGET

N- and S-substituted derivatives of 2-aminobenzenethiol were prepared as intermediates in the synthesis of analogs of the phenothiazine tranquilizers. A new synthesis for the preparation of the benzothiazine ring is reported. The ring cleavage of 2,2-pentamethylenebenzothiazoline with alkylating agents is discussed.

IN RECENT years derivatives of 2-aminobenzenethiol have attracted attention in the development of medicinal agents. Gialdi and Baruffini (1) synthesized a series of 2-aminophenyl alkyl sulfides as potential fungicides. Burger, *et al.* (2), prepared N-substituted 2-aminophenyl aryl sulfides and found some of them to be radiation protective agents. We have undertaken the synthesis of analogs of the phenothiazine tranquilizers and found it necessary to prepare N- and S-substituted 2-aminobenzenethiols as intermediates. Bialdi and Baruffini (3) had prepared similar thiols by reducing 2,2′-dinitrodiphenyldisulfide with glucose to 2-nitrobenzenethiol. 2-Nitrobenzenethiol was then substituted with an alkyl bromide to give 2-nitrophenyl alkyl sulfide. This paper reports a one-step procedure of reacting commercial 2-aminobenzenethiol with alkyl halide in the presence of potassium hydroxide solution. Not only are the compounds prepared in fewer steps than those of Gialdi and Baruffini, but the yields of the corresponding products are

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