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STUDIES OF THE CHEMICAL NATURE OF THE α-ADRENERGIC RECEPTOR—III

FURTHER INVESTIGATION OF THE LABELING PROCEDURE*

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Abstract—After exposing rabbit aortic strips to ^{14}C-2-dibenzylaminoethanol hydrochloride, all the radioactivity could be removed by washing for 6 hr. This observation showed that the retention of radioactivity by aortic strips exposed to ^{14}C-Dibenamine hydrochloride is not due to binding of its hydrolysis product, viz. ^{14}C-2-dibenzylaminoethanol. The ^{14}C-Dibenamine hydrochloride remaining in rabbit aortic strips after washing is not exchangeable with unlabeled Dibenamine hydrochloride. ^{14}C-Dibenamine hydrochloride was found to be distributed between all subcellular components of rabbit aorta. Since α-adrenergic receptor blockade could be achieved in experiments in which ^{14}C-Dibenamine hydrochloride was located exclusively in the lipid-free residues, a lipid is probably not involved in the α-adrenergic receptor sites. Uptake of ^{14}C-Dibenamine hydrochloride in rabbit aortic strips could not be demonstrated without the observance of a concomitant decline in the maximum response attainable to epinephrine, suggesting the absence of a significant population of spare receptors.

HARVEY and Nickerson[1] attributed the pharmacological activity of Dibenamine and its congeners to the formation at physiological pH of a highly reactive and unstable ethylenimmonium (EI) ion. This idea was based on the known formation of EI ions by the structurally related nitrogen mustards. Since it is well known that EI ions are highly reactive substances and react readily with nucleophilic groups, Nickerson[2, 3] interpreted the prolonged duration of the α-adrenergic blockade produced by Dibenamine and its congeners to the formation of a covalent bond between Dibenamine and the α-adrenergic receptor. Moran *et al.*[4] studied the distribution of ^{3}H-*N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthylmethylamine (^{3}H-SY·28) and ^{3}H-*N*-(2-hydroxylethyl)-*N*-ethyl-*N*-1-napthylmethylamine (^{3}H-SY·28 alcohol) in various organs of mice following intravenous (i.v.) injection of these compounds. On the basis of these studies it was suggested that the prolonged tissue retention of radioactivity after treatment with ^{3}H-SY·28 was not due to covalent binding but rather to the high-binding capacity of tissue for the alcohol derived by hydrolysis of ^{3}H-SY·28. In our previous studies on the α-adrenergic receptor[5, 6] we have utilized ^{14}C-Dibenamine hydrochloride which belongs to the same chemical class as ^{3}H-SY·28, viz. the 2-halogenoethylamines. The success of our procedure is based on the assumption that ^{14}C-Dibenamine hydrochloride forms a covalent bond with the α-adrenergic receptor. It was therefore important to demonstrate that the prolonged retention of radioactivity observed after treatment of rabbit aortic strips with ^{14}C-Dibenamine

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hydrochloride was not due to the high-binding capacity of aortic strips for 2-dibenzylaminoethanol, the alcohol derived by hydrolysis of Dibenamine hydrochloride. For this reason ^{14}C-labeled 2-dibenzylaminoethanol hydrochloride was prepared and the binding of this compound to rabbit aortic strips studied.

Takagi *et al.*[7] labeled the acetylcholine receptors of intestinal smooth muscle with ^{3}H-labeled Dibenamine as follows: The receptors were partially protected with atropine, while other receptors were masked with unlabeled Dibenamine hydrochloride; after washing, the acetylcholine receptors were labeled with ^{3}H-Dibenamine hydrochloride. They reported that subcellular fractionation of the labeled tissue was useful as a preliminary procedure for the isolation of the ^{3}H-Dibenamine-acetylcholine receptor complex. For this reason we have labeled the α-adrenergic receptor of rabbit aortic strips with ^{14}C-Dibenamine hydrochloride and undertaken the separation of this tissue into its subcellular components.

The relationship between the amount of ^{14}C-Dibenamine hydrochloride taken up by aortic strips and the degree of blockade produced was examined with two aims in mind: (1) to see if a degree of blockade of the α-adrenergic receptors could be achieved without labeling of the lipid components of this tissue and thus eliminate the possibility that lipid is a component of these receptors; (2) to see whether ^{14}C-Dibenamine hydrochloride is taken up by the strips prior to the achievement of a degree of blockade of the α-adrenergic receptors and thus obtain direct evidence for the existence of spare receptors in this tissue.

EXPERIMENTAL

The infra-red spectrum was obtained with a Perkin–Elmer 137 sodium chloride spectrophotometer; 2-dibenzylaminoethanol hydrochloride was mulled with Nujol. The melting point is uncorrected. Probability values (P) were obtained by means of a one-tailed *t*-test.

Preparation of rabbit aortic strips. Rabbits weighing between 1·5 and 3 kg were killed by a blow on the back of the neck. The descending thoracic aorta was quickly removed and placed in a Petri dish containing Krebs bicarbonate solution[5] aerated with 95 per cent oxygen and 5 per cent carbon dioxide. After removing the adipose and connective tissue from the aorta, a helical strip was prepared according to Furchgott and Bhadrakom[8] and mounted as single strips in organ baths of 15-ml working volume with the aid of thread and stainless-steel hooks. The responses of the tissue were recorded by means of force and displacement transducers (Grass FT·03) connected to a Grass model 5P1 polygraph. The loading tension applied to the strips was 2 *g*. The volume of Krebs bicarbonate solution in the organ bath was 14·8 ml. Drugs were made up to a final volume of 0·2 ml before addition to the bath. The following drugs were used: *l*-epinephrine bitartrate (Nutritional Biochemicals); Dibenamine hydrochloride (Smith, Kline & French); ^{14}C-Dibenamine hydrochloride (sp. act. 0·48 mc/m-mole). The final drug concentrations, unless otherwise specified, are expressed as grams of salt per milliliter bath fluid.

Determination of radioactivity of lipid and lipid-free residue of aortic strips. Strips containing radioactivity were dried from the frozen state, weighed and the lipids extracted twice with chloroform–methanol (2:1) at room temperature. A portion of the chloroform–methanol extract (1 ml) was transferred to a counting vial and the solvent removed by aeration. The residue was dissolved in 18 ml of counting solution

and the radioactivity determined in a liquid scintillation counter (Nuclear Chicago unilux model 6850). The lipid-free residue was dissolved at 70° in 1 ml of 5 N KOH/50 mg original tissue; 0·2-ml aliquots were added to 17·8 ml of counting solution and the radioactivity determined.[5]

Preparation of ^{14}C-labeled-2-dibenzylaminoethanol hydrochloride. ^{14}C-2-Dibenzylaminoethanol hydrochloride was prepared by a method previously used to prepare the unlabeled compound.[9] Benzyl-7-^{14}C-chloride (0·25 g; 2 m-mole; New England Nuclear Corp.) was treated with 2-aminoethanol (0·122 g; 2 m-mole) and the product distilled (bulb-tube), affording ^{14}C-2-dibenzylaminoethanol (0·146 g; 65%) as a colorless oil, b.p. 125–130°/0·03 mm. The product was dissolved in ethanol and the solution saturated with hydrogen chloride. Upon adding a few drops of ether, a precipitate was obtained which was recrystallized from ethanol affording ^{14}C-2-dibenzylaminoethanol hydrochloride (sp. act. 0·28 mc/m-mole) as white crystals (0·1 g; 57%) m.p. 171–173°. When mixed with 2-dibenzylaminoethanol hydrochloride (Aldrich Chemical Co.) it had a m.p. of 171–173°. The infra-red spectrum of the ^{14}C-2-dibenzylaminoethanol hydrochloride was identical in all respects with that of 2-dibenzylaminoethanol hydrochloride. To ensure that ^{14}C-2-dibenzylaminoethanol hydrochloride was radiochemically pure it was recrystallized twice more from ethanol and had a specific activity of 0·32 mc/m-mole.

Removal of loosely bound ^{14}C-2-dibenzylaminoethanol hydrochloride from rabbit aortic strips. The responses of eight rabbit aortic strips to several concentrations of epinephrine were recorded and the strips then exposed to ^{14}C-2-dibenzylaminoethanol hydrochloride ($2{\cdot}8 \times 10^{-6}$) for 20 min. Strips 1 and 2 were removed from the organ baths without washing, and the remaining strips were washed with Krebs bicarbonate solution at 15-min intervals. Strips 3 and 4 were removed from the organ baths after 1 hr, strips 5 and 6, after 3 hr, and strips 7 and 8 after 6 hr. The radioactivity in lipid extracts and lipid-free residues was then determined.

The effect of unlabeled Dibenamine hydrochloride on the retention of ^{14}C-Dibenamine hydrochloride by rabbit aortic strips. The response of four aortic strips to epinephrine was recorded. Two of the strips were exposed to ^{14}C-Dibenamine hydrochloride (3×10^{-6}) for 20 min and the strips then washed with Krebs bicarbonate solution containing unlabeled Dibenamine hydrochloride (3×10^{-6}) at 15-min intervals. Because of the instability of Dibenamine hydrochloride, fresh solutions of the agent in Krebs bicarbonate solution were made up at 15-min intervals. One strip was removed from an organ bath after 1 hr and a second strip after 4 hr; the radioactivity in the lipid extracts and lipid-free residues of the strips was determined. As a control the above experiment was repeated with the omission of unlabeled Dibenamine hydrochloride (3×10^{-6}) from the Krebs bicarbonate wash fluid using the remaining two strips.

Labeling the α-adrenergic receptor of rabbit aortic strips. The responses of rabbit aortic strips to several concentrations of epinephrine were recorded. The strips were treated with epinephrine bitartrate (1×10^{-4}) for 5 min and ^{14}C-Dibenamine hydrochloride (3×10^{-6}) added. After a further 20-min period the strips were washed for 3 hr at 15-min intervals. Strips treated as above have been designated experimental strips. As controls, the experiments were repeated with the omission of the protecting dose of epinephrine bitartrate (1×10^{-4}) in the first step. These strips were used for the subcellular fractionation studies described below.

Preparation of subcellular fractions of rabbit aorta. The procedure of Whereat[10] was used for isolation of the subcellular components of rabbit aorta at 4°. A fine mince was prepared from labeled rabbit aortic strips with the aid of a pair of small scissors. The minced tissue was placed in the upper bowl of an all glass TenBroeck tissue grinder (Kontes Glass Co.) and 30 ml of 0·28 M sucrose solution (containing 5×10^{-5} M EDTA) per gram of minced tissue was added to the barrel. The homogenate H was centrifuged at 800 *g* for 6 min and the supernatant transferred to a second tube. The residue, containing nuclei, was resuspended in fresh 0·28 M sucrose solution and centrifuged at 800 *g* for 6 min. The combined supernatant MES was centrifuged at 10,000 *g* for 15 min in a Beckman model L 4 ultracentrifuge. The residue, containing mitochondria, was resuspended in fresh 0·28 M sucrose solution and recentrifuged at 10,000 *g* for 15 min. The combined supernatant ES was centrifuged at 100,000 *g* for 90 min. The supernatant S was removed leaving a residue containing microsomes.

Determination of the protein content and radioactivity of each subcellular fraction. Since only small amounts of material were available the following indirect procedure was used for the determination of protein content and radioactivity of each subcellular fraction: the protein content and radioactivity of measured aliquots of the whole homogenate H, supernatant MES, supernatant ES, and the supernatant S were determined. The protein content and radioactivity associated with the nuclear fraction represents the difference between the protein content and radioactivity of homogenate H and supernatant MES and thus could be calculated. In a similar way, the protein content and radioactivity associated with the mitochondrial and microsomal fractions were calculated.

The method of Lowry, as modified by Miller,[11] was used for the protein determination. The radioactivity associated with homogenate H and supernatant fractions, MES, ES and S was determined as follows: the samples (0·1 ml) were digested at 70° in 5 N KOH solution (0·1 ml). For use as a blank the 0·28 M sucrose (0·1 ml) was digested

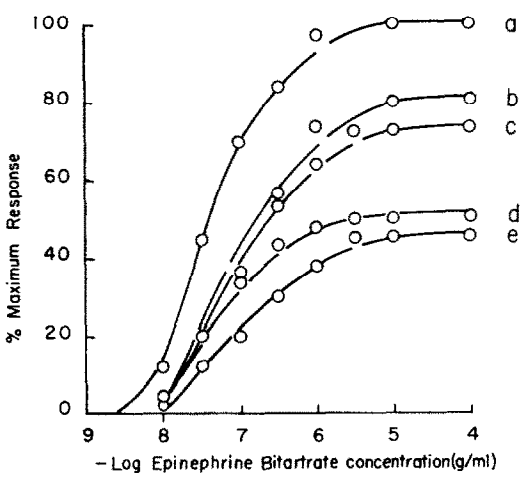

FIG. 1. Response of rabbit aortic strips to epinephrine after exposure to the following concentrations of ^{14}C-Dibenamine hydrochloride for 20 min: a, control (no ^{14}C-Dibenamine hydrochloride added); b, 3×10^{-8}; c, 6×10^{-8}; d, 9×10^{-8}; e, $1{\cdot}2 \times 10^{-7}$. Each point represents the mean of twelve independent observations in a, and three independent observations in b, c, d and e.

with 5 N KOH solution (0·1 ml). Reagent grade methanol (5·3 ml) and scintillation solution (12·5 ml) were added to the cooled digests. The scintillation solution was prepared by dissolving 2,5-diphenyloxazole (PPO; 6 g) and 2-*p*-phenylene-*bis*(5-phenyl oxazole) (POPOP; 0·1 g) in reagent grade toluene (1 liter).

Relationship between uptake of ^{14}*C-Dibenamine hydrochloride and blockade of α-adrenergic receptors.* (a) The responses of twelve rabbit aortic strips to several concentrations of epinephrine were recorded. From the average of these responses a cumulative dose–response curve was constructed (Fig. 1 a). Aortic strips were then exposed to the following concentrations of ^{14}C-Dibenamine hydrochloride for 20 min: strips 1, 2 and 3, 3×10^{-8}; strips 4, 5 and 6, 6×10^{-8}; strips 7, 8 and 9, 9×10^{-8}; and strips 10, 11 and 12, $1{\cdot}2 \times 10^{-7}$. The strips were washed for 3 hr at 15-min intervals and cumulative dose–response curves to epinephrine were redetermined (Fig. 1 b, c, d, e).

(b) The above experiment was repeated with the following modifications in concentration and time of exposure of aortic strips to ^{14}C-Dibenamine hydrochloride; strips 1 and 2, $1{\cdot}2 \times 10^{-7}$ for 2 min (Fig. 2 b); strips 3 and 4, $1{\cdot}2 \times 10^{-7}$ for 4 min (Fig. 2 c); strips 5 and 6, $1{\cdot}2 \times 10^{-7}$ for 6 min (Fig. 2 d); strips 7 and 8, 3×10^{-6} for 2 min (Fig. 2 e); strips 9 and 10, 3×10^{-6} for 4 min (Fig. 2 f) and strips 11 and 12,

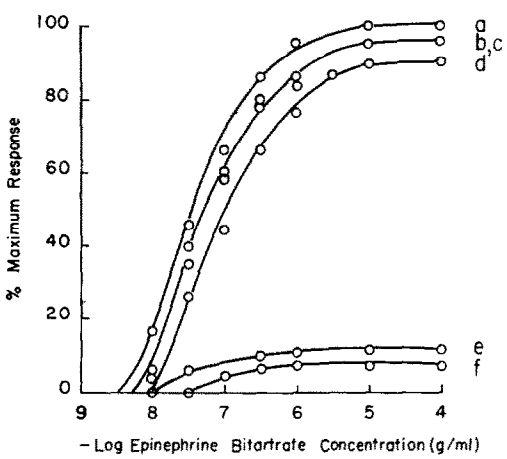

FIG. 2. Response of rabbit aortic strips to epinephrine after exposure to the following concentrations of ^{14}C-Dibenamine hydrochloride for various time periods: a, control (no ^{14}C-Dibenamine hydrochloride added); b, $1{\cdot}2 \times 10^{-7}$ for 2 min; c, $1{\cdot}2 \times 10^{-7}$ for 4 min; d, $1{\cdot}2 \times 10^{-7}$ for 6 min; e, 3×10^{-6} for 2 min; f, 3×10^{-6} for 4 min. Each point represents the mean of twelve independent observations in a, and two independent observations in b, c, d, e and f.

3×10^{-6} for 6 min. The tissue was unresponsive to epinephrine after exposure to ^{14}C-Dibenamine hydrochloride (3×10^{-6}) for 6 min.

The amount of radioactivity associated with lipid extracts and lipid-free residues of the strips described in (a) and (b) was determined.

RESULTS AND DISCUSSION

The results (Fig. 3) show that after exposing strips to ^{14}C-2-dibenzylaminoethanol hydrochloride all the radioactivity can be removed by washing for 6 hr at 15-min

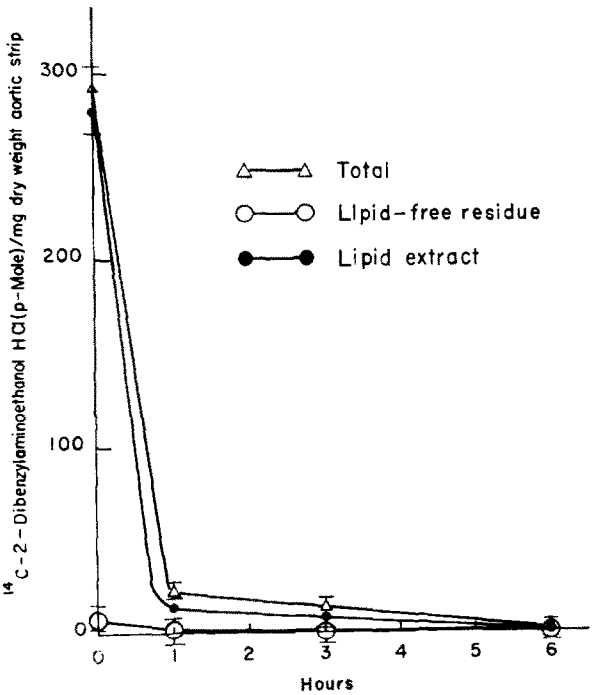

FIG. 3. Removal by washing of radioactivity from rabbit aortic strips after exposure to ^{14}C-2-dibenzylaminoethanol hydrochloride ($2{\cdot}8 \times 10^{-6}$) for 20 min. Vertical lines represent $\pm$ S.D. of the mean. Each point represents the mean of two to four independent observations.

intervals. In a similar experiment previously carried out with ^{14}C-Dibenamine hydrochloride, (3×10^{-6}) only 50 per cent of the radioactivity could be removed.[6] The results of the previous experiment are shown in Fig. 4 in order to facilitate comparison with the present results. The prolonged retention of radioactivity following exposure of aortic strips to ^{14}C-Dibenamine hydrochloride is clearly not due to the high binding capacity of strips for the alcohol which would be obtained upon hydrolysis of Dibenamine hydrochloride, viz. 2-dibenzylaminoethanol. The above finding does not, however, invalidate the previous observations of Moran *et al.*[4] regarding the high-binding capacity of rat tissues for ^{3}H-SY·28 alcohol following i.v. injection of this compound. This conclusion follows from the wide difference in technique employed in our present study as compared to that used by Moran *et al.*[4] The results recorded in Table 1 show that addition of unlabeled Dibenamine hydrochloride to the wash fluid does not result in a lowered retention of radioactivity by rabbit aortic strips which have been exposed to ^{14}C-Dibenamine hydrochloride. Thus the ^{14}C-Dibenamine hydrochloride retained by the strips is not exchangeable with unlabeled Dibenamine hydrochloride in the bath fluid. This finding indicates that the radioactivity retained in the rabbit aortic strips after exposure to ^{14}C-Dibenamine hydrochloride represents that portion of this agent covalently bound to tissue components.

The results recorded in Table 2 show that the radioactivity is distributed between all subcellular components. Moreover, significant differences exist between the

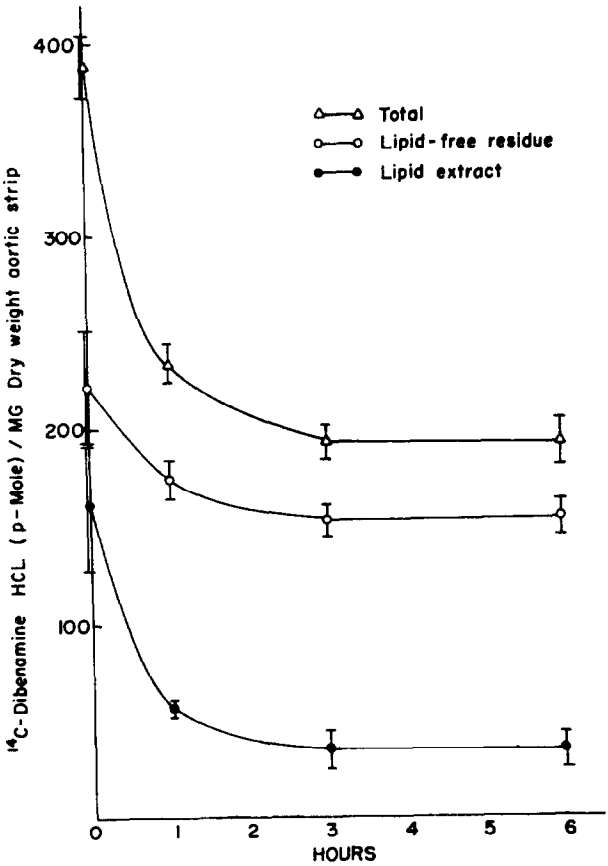

FIG. 4. Removal by washing of radioactivity from rabbit aortic strips after exposure to ^{14}C-Dibenamine hydrochloride (3×10^{-6}) for 20 min. Vertical lines represent ± S.D. of the mean. Each point represents the mean of two to four independent observations.[6]

TABLE 1. EFFECT OF WASHING ^{14}C-DIBENAMINE HYDROCHLORIDE-LABELED AORTIC STRIPS WITH UNLABELED DIBENAMINE HYDROCHLORIDE SOLUTION

Aortic strip	Total dpm/mg dry weight of strip ± S.D.	
	1 hr	4 hr
Wash with Krebs bicarbonate solution	311 ± 63 (6)*	178 ± 18 (4)
Wash with 3×10^{-6} g/ml of Dibenamine HCl	299 ± 70 (6)	217 ± 67 (4)
P value	> 0·1	> 0·1

* Numbers enclosed by parentheses indicate the number of experiments performed.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN SUBCELLULAR FRACTIONS OF CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS*

Fraction	Protein content ± S.D. (mg)		Total dpm ± S.D.		Dpm/mg protein ± S.D.		C − E† (%)
	C	E	C	E	C	E	E
Homogenate	7·8 ± 1·7	8·2 ± 1·4	5511 ± 1404	3977 ± 1238	696 ± 69	486‡ ± 85	43·2
Nuclear	4·2 ± 1·1	5·1 ± 1·5	2706 ± 792	2247 ± 631	618 ± 149	486‡ ± 85	27·2
Mitochondrial	2·6 ± 0·7	2·4 ± 0·5	1917 ± 164	1043 ± 422	722 ± 195	475‡ ± 85	52·0
Microsomal	0·5 ± 0·1	0·4 ± 0·1	490 ± 171	209 ± 82	1072 ± 274	582‡ ± 182	84·2
Supernatant	0·4 ± 0·08	0·5 ± 0·01	328 ± 152	330 ± 174	781 ± 242	721 ± 277	8·3

* The results reported represent the mean of six independent experiments.
† C = control; E = experimental.
‡ Significant difference at 0·05 level.

radioactivity in all particulate components of control strips when compared to the same particulate components of experimental strips. It was of interest that the largest difference in radioactivity was found in the microsomal fraction and the second largest difference in the mitochondrial fraction, a result which corresponds to that of Takagi *et al.*[7] in labeling studies on acetylcholine receptors. The latter workers, on the basis of their findings of a large difference in radioactivity in the mitochondrial and microsomal fractions, combined these fractions and used them in their subsequent fractionation studies, ignoring the radioactivity associated with other fractions. A careful examination of our data leads to the conclusion that it is unreasonable to proceed on this basis and ignore the radioactivity associated with the nuclear and the supernatant S fraction. This is particularly apparent in our data where approximately 50 per cent of the total radioactivity is associated with the nuclear fraction. One interpretation of our results is that the subcellular fractions are impure and a ^{14}C-Dibenamine-receptor complex, normally present in a particular location in the cell is contaminating the other fractions. Whereat,[10] who devised the fractionation procedure, was concerned primarily with isolating the mitochondrial fraction. Consequently, although he was able to demonstrate the relative purity of the mitochondrial fraction, no evidence is available regarding the purity of the other fractions. A second interpretation of our results is that ^{14}C-Dibenamine, in addition to labeling the α-adrenergic receptors located in a particular cellular component, is also labeling sites in other subcellular components. In view of the above observations and the difficulty in locating the ^{14}C-Dibenamine-α-adrenergic receptor complex in a particular subcellular component this approach to the problem was not pursued further.

The data presented in Table 3 were utilized to construct the curve (Fig. 5) which shows the relationship between the amount of ^{14}C-Dibenamine hydrochloride taken up by rabbit aortic strips and the maximum epinephrine responses attainable. It was of interest that a considerable degree of irreversible α-adrenergic blockade (approximately 50 per cent) could be achieved with the ^{14}C-Dibenamine hydrochloride

TABLE 3. RELATIONSHIP BETWEEN THE UPTAKE OF ^{14}C-DIBENAMINE HYDROCHLORIDE AND THE BLOCKADE OF α-ADRENERGIC RECEPTORS IN RABBIT AORTIC STRIPS

Concentration of ^{14}C-Dibenamine HCl (g/ml)	Exposure time (min)	^{14}C-Dibenamine HCl (p-mole)/mg dry weight of strip*			% Maximum response to epinephrine
		Lipid extract	Lipid-free residue	Total	
3×10^{-8}	20	0	2·8	2·8	80
6×10^{-8}	20	0	3·8	3·8	75
9×10^{-8}	20	0	8·1	8·1	50
$1{\cdot}2 \times 10^{-7}$	2	0	1·8	1·8	95
	4	0	1·8	1·8	90
	6	0	2·8	2·8	80
	20	0·9	9·9	10·8	45
3×10^{-6}	2	4·7	29·2	34·0	12
	4	10·4	52·8	63·2	8
	6	15·1	63·2	78·3	0

* Each figure represents the mean of three independent observations.

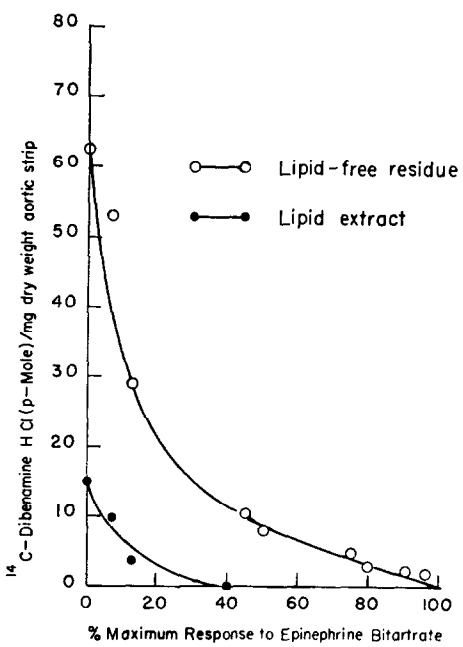

FIG. 5. Relationship between the uptake of ^{14}C-Dibenamine hydrochloride and the blockade of α-adrenergic receptors in rabbit aortic strips.

located exclusively in the lipid-free residue. This observation, which is in accord with the observations of Lewis and Miller,[12] shows that it is unlikely that a lipid component is involved in the α-adrenergic receptor sites, as suggested by the studies of Dikstein and Sulman.[13]

The basic assumption of Clark[14] that intensity of tissue response is directly related

to the number of receptors occupied by agonists has been challenged by Furchgott,[15] Stephenson[16] and Nickerson,[17] who showed that maximum drug effects could be observed when only a small percentage of available receptors was occupied. It was suggested that maximal drug effects could be obtained in certain tissues when only 1 per cent of the available receptors was occupied and the remaining receptors were referred to as "spare receptors". If spare receptors do exist in tissues it should be possible to demonstrate the uptake of ^{14}C-Dibenamine hydrochloride or other irreversible agents of this type by these spare receptors without the observance of a concomitant decline in the maximum response attainable to epinephrine.

In Fig. 5 it is seen that the maximum response to epinephrine is immediately reduced upon uptake of a detectable amount of ^{14}C-dibenamine hydrochloride, suggesting that there is not a significant population of spare receptors in rabbit aorta. We believe that this labeling technique will prove to be of value in obtaining direct evidence for the existence of spare receptors, in tissues where they are believed to exist. It is noteworthy that May *et al.*[18] using the aortic strip, and Lewis and Miller[12] using the seminal vesicle have, by means of different techniques, provided evidence for the absence of a significant population of spare receptors in these tissues.

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