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STUDIES OF THE CHEMICAL NATURE OF THE α-ADRENERGIC RECEPTOR*

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Abstract—^{14}C-Labeled dibenamine hydrochloride was prepared and used to label the α-adrenergic receptor of rabbit aortic tissue. Approximately one third of the ^{14}C-labeled dibenamine hydrochloride was located in the lipid extract of aortic tissue, and the distribution of radioactivity in the lipid components has been examined. No evidence could be found to support the claim that cephalin serves as a tissue acceptor for dibenamine hydrochloride.

ACCORDING to a recent study,[1] the prolonged blockade of the α-adrenergic receptor by members of the dibenamine group of blocking agents[2] is interpreted as follows. One of the carbon atoms of the ethylenimmonium ion, generated *in vivo* from members of this group of blocking agents, is positively charged and enters into covalent bond formation with an anionic site on the α-adrenergic receptor. This concept suggested an approach whereby information on the chemical nature of the α-adrenergic receptor might be obtained. In the present study ^{14}C-labeled N-(2-chlorethyl)-dibenzylamine hydrochloride (dibenamine hydrochloride) was prepared and used to label the α-adrenergic receptor of rabbit aortic strips. The labeled strips were subjected to chemical fractionation and the distribution of radioactivity in several tissue components studied. While this work was in progress, a report appeared of a similar study of dibenamine hydrochloride randomly labeled with tritium. In this report[3, 4] it was suggested that cephalin is the tissue acceptor for dibenamine. For this reason, in the present study emphasis was placed on the distribution of radioactivity in the lipid components of the tissues.

EXPERIMENTAL

The infrared spectrum was obtained with a Perkin Elmer 137 sodium chloride spectrophotometer; dibenamine 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 ^{14}C-labeled dibenamine hydrochloride

Dibenamine hydrochloride has been prepared by a method previously used by Gump and Nikawitz[5] to prepare the unlabeled compound. Benzyl-7-^{14}C-chloride (0·5 g; 4 m-moles; 0·25 mc/m-mole; New England Nuclear Corp.) was treated with 2-aminoethanol (0·244 g; 4 m-moles) and the product distilled (bulb-tube), affording 2-dibenzylaminoethanol (0·41 g; 86%) as a colorless oil, b.p. 130°/0·03 mm. The

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2-dibenzylaminoethanol (0·41 g; 1·71 m-moles) was treated with thionyl chloride (0·28 g; 2·34 m-moles) in chloroform (0·4 ml). The product was recrystallized from ethanol, affording ^{14}C-labeled dibenamine hydrochloride as white crystals (0·285 g; 57%), m.p. 188–190°. When mixed with a sample of dibenamine hydrochloride [Smith, Kline and French (SKF)] it had m.p. 188–190°. The infrared spectrum of the ^{14}C-labeled dibenamine hydrochloride was identical in all respects with that of dibenamine hydrochloride (SKF). It had a specific activity of 0·48 mc/m-mole.

Labeling the α-adrenergic receptor of rabbit aortic tissue

Procedure IA. Rabbit aortic strips, prepared as described by Furchgott,[6] were mounted in muscle chambers of 15-ml working volume and the contractions recorded by means of force and displacement transducers (Statham model G7A) connected to a Grass model 5 polygraph. The loading tension applied to the strips was 2 g. The aortic strips were immersed in a solution containing 0·116 M NaCl, 0·0046 M KCl, 0·0024 M $CaCl_2 \cdot 2H_2O$, 0·001 M $MgSO_4 \cdot 7H_2O$, 0·001 M $NaH_2PO_4.H_2O$, 0·021 M $NaHCO_3$, and 0·045 M dextrose; the solution was aerated with 5% CO_2–95% O_2 at 37°. The following drugs were used: l-epinephrine (l-adrenaline) bitartrate (Nutritional Biochemicals); histamine phosphate (Fischer Scientific); serotonin (5-hydroxytryptamine creatinine sulfate, Nutritional Biochemicals); acetylcholine bromide (Eastman Organic Chemicals); and dibenamine hydrochloride (SKF). The final drug concentrations are expressed as grams of salt per milliliter bath fluid. The responses of the aortic strips to several concentrations of adrenaline, histamine, serotonin, and acetylcholine were recorded. The strips were then treated with adrenaline (1×10^{-4}) for 5 min and unlabeled dibenamine hydrochloride (3×10^{-6}) added. After a further 20 min period the strips were washed three times with adrenaline (1×10^{-4}) and three times with bath fluid. The strips were washed with bath fluid at 15 min intervals until they relaxed completely, and the response to various concentrations of adrenaline and other drugs were redetermined. The remaining sensitivity of the strips to drugs as a percentage of the original sensitivity was calculated according to Furchgott.[6] As expected, the strips retained considerable responsiveness to adrenaline, slight responsiveness to acetylcholine, but were unresponsive to serotonin and histamine. The strips were exposed to ^{14}C-labeled dibenamine hydrochloride (3×10^{-6} for 20 min and then washed six times with bath fluid. After this treatment the strips were unresponsive to adrenaline, showing that the α-adrenergic receptor had interacted with ^{14}C-labeled dibenamine hydrochloride. Strips treated as above have been designated experimental strips. As controls, the experiments were repeated, omitting the protecting dose of adrenaline (1×10^{-4}) in the first step.

Procedure IB. The above experiments were repeated with adrenaline ($3{\cdot}3 \times 10^{-4}$) in the first step of the procedure, followed by unlabeled dibenamine hydrochloride (1×10^{-5}). In these experiments the responses of the tissue to histamine, serotonin, and acetylcholine were not studied.

Procedure II. The responses of rabbit aortic strips to several concentrations of adrenaline were recorded. The strips were treated with adrenaline (1×10^{-4}) for 5 min and ^{14}C-labeled dibenamine hydrochloride (3×10^{-6}) added. After a further 20-min period the strips were washed as in procedure IA, allowed to relax completely, and the

response to various concentrations of adrenaline redetermined. Strips treated as above have been designated experimental strips. As controls, the experiments were repeated with the omission of the protecting dose of adrenaline (1×10^{-4}) in the first step.

Determination of total radioactivity in experimental and control aortic strips

Aortic strips labeled with ^{14}C-dibenamine hydrochloride by procedures IB and II were dried from the frozen state, weighed, and dissolved at 70° in 1 ml 5 N KOH/50 mg tissue; 0·2 ml aliquots were added to 17·8 ml of counting solution[7] and the radioactivity determined in a liquid scintillation counter (Nuclear Chicago, liquid scintillation system 724–725). All samples were counted for 40 min and background for 80 min. These time periods were calculated to be long enough to bring the percentage standard error for the corrected count down to $\pm 2\%$ except in those cases in which only traces of radioactivity were detected on the chromatogram. A curve was plotted of per cent counting efficiency vs. the L_3–L_4/L_3–L_5 channel ratio by using a series of sealed ^{14}C-quenched standards. By means of this curve the counting efficiency for each sample was determined from the value of its channels ratio. The counting efficiency was found to be in the range of 60% to 75%.

Determination of radioactivity in lipid and lipid-free residue of experimental and control aortic strips

Strips labeled with ^{14}C-dibenamine hydrochloride by procedures IA and II 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 (0·8 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. The lipid-free residue was dissolved at 70° in 1 ml 5 N KOH/50 mg original tissue; 0·2-ml aliquots were added to 17·8 ml of counting solution and the radioactivity determined.

Investigation of the lipid extracts of aortic strips by thin-layer chromatography

A part of the extract was purified according to the method of Folch *et al.*[8] and concentrated to 0·3 ml by removing the solvent under reduced pressure at room temperature. Chromatoplates (20 × 10 cm) were prepared by a modification of the procedure described by Skipski *et al.*[9], with a slurry of silica gel G (10 g) in 0·01 M sodium acetate (27 ml). The following substances were applied to each chromatoplate. (1) purified lipid extracts of rabbit aortic strips; (2) purified lipid extracts of experimental strips; (3) purified lipid extracts of control strips; (4) ^{14}C-labeled dibenamine hydrochloride; (5) sphingomyelin (purified from beef brain, Sigma Chemical); (6) phosphatidyl-L-serine (fraction III of Folch, from bovine brain, grade II, Sigma Chemical); (7) L-α-cephalin (β,γ-dipalmitoyl) synthetic (lyso-free, B grade, Calbiochem) or phosphatidylethanolamine (fraction V of Folch, from bovine brain, grade II, Sigma Chemical); (8) a mixture of (5), (6) and (7). The chromatograms were developed with a mixture of chloroform:methanol:water (75:22:3)[4] and the individual compounds detected by means of iodine vapour. The plates were exposed to the atmosphere until the brown spots were decolorized and segments of the chromatogram scraped into small columns. The columns were eluted with 3 ml of chloroform:methanol (2:1)

and the eluate evaporated to dryness. Counting solution (18 ml) was added to dissolve the residue and the radioactivity determined.

RESULTS AND DISCUSSION

Furchgott[6] had identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta, which are specific for histamine, acetylcholine, serotonin, and adrenergic drugs respectively. He furthermore demonstrated that when this preparation is exposed to adrenaline (1×10^{-4}) for 5 min and dibenamine hydrochloride (3×10^{-6}) added, the latter drug combines with the histamine, serotonin, and acetylcholine receptor, respectively, while the adrenaline receptor is protected, at least partially, from combination with this drug. In our experiments this method has been used to mask the histamine, serotonin, and acetylcholine receptors with unlabeled dibenamine hydrochloride; the adrenaline receptor which was protected under these conditions was then labeled with ^{14}C-labeled dibenamine hydrochloride. After labeling the α-adrenergic receptor with ^{14}C-labeled dibenamine hydrochloride by this method (procedure IA) and a modification of this method (procedure IB), it was expected that the experimental strips would contain significantly greater radioactivity than the control strips. It was desirable to determine that this was in fact the case, and the results obtained in nine experiments with procedure IA and eight experiments with procedure IB are recorded in Tables 1 and 3. A significant increment in radioactivity

TABLE 1. THE DISINTEGRATIONS PER MINUTE DETECTED IN CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS LABELED BY PROCEDURE IB

Remaining sensitivity to adrenline (% of original)	Dry weight of aortic strip (mg)		Total dis/mn/mg of dry weight of strip	
	Control strip	Experimental strip	Control strip	Experimental strip
20	17·3	16·1	106	201
33	16·7	17·3	182	208
19	14·2	12·3	124	178
11	21·3	22·7	125	122
17	23·0	20·4	151	216
12	14·4	17·5	146	298
16	20·1	25·0	233	208
9	11·7	13·8	176	212
Mean ± S.D.	17·3 ± 3·6	18·1 ± 4·1	155 ± 38	205 ± 45
P Value			<0·025	

($P < 0{\cdot}05$, procedure IA; $P<0{\cdot}025$, procedure IB) was noted in the experimental strips as compared to the control strips. This increment in radioactivity represents that portion of the ^{14}C-labeled dibenamine hydrochloride combined with sites protected by adrenaline, which might be in large part α-adrenergic receptor sites. An alternative procedure for labeling and studying the α-adrenergic receptor is described in procedure II in the experimental section. With this procedure it was anticipated that the control strips would contain significantly greater radioactivity than the experimental strips. The results obtained in five experiments with procedure II are recorded in Table 2. A significant increment in radioactivity ($P<0{\cdot}01$) was noted

in the control strips as compared to the experimental strips. Of the three labeling procedures used, the most significant differences in radioactivity between control and experimental strips was obtained by procedure II.

Experimental and control strips labeled by procedure IA and II were subjected to chemical fractionation with the object of isolating a ^{14}C-labeled dibenamine-α-adrenergic receptor complex. This complex should possess the following characteristics: it should be a substance isolated from the experimental strips with a higher ^{14}C-content than a corresponding substance isolated from the control strips (procedure IA),

TABLE 2. THE DISINTEGRATIONS PER MINUTE DETECTED IN CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS, LABELED BY PROCEDURE II

Remaining sensitivity to adrenaline (% of original)	Dry weight of aortic strip (mg)		Total dis/min/mg of dry weight of strip	
	Control strip	Experimental strip	Control strip	Experimental strip
38	15·4	10·7	171	137
43	13·2	10·9	156	99
20	16·5	15·9	179	151
41	13·1	12·0	212	158
40	17·0	19·0	185	142
Mean ± S.D.	15·2 ± 1·6	13·7 ± 3·2	181 ± 19	137 ± 21
P Value			<0·01	

and for this reason should be identifiable. Conversely, with labeling procedure II, it should be a substance isolated from the control strips with a higher ^{14}C-content than a corresponding substance isolated from the experimental strips. In view of the recent suggestion that cephalin is the tissue acceptor for dibenamine[3,4] it was of interest to determine the amount of radioactivity in the lipid extracts and lipid-free residues of the aortic strips. The results obtained in nine experiments (procedure IA) and seven experiments (procedure II) are recorded in Tables 3 and 4 respectively. A significant increment in radioactivity ($P<0·005$) was noted in the lipid extracts of control strips as compared to the lipid extracts of experimental strips labeled according to procedure II. However, no significant difference in radioactivity was found on comparing the lipid extracts of control and experimental strips labeled by procedure IA. The results obtained from procedure II are in agreement with those obtained by Dikestein and Sulman,[4] who used an analogous procedure.

Of considerable interest was the finding that only 20–23% of the ^{14}C-content of the strip was located in the lipid extracts (procedure II). This finding differs from the report of Dikstein *et al*[3]. who, using a similar labeling procedure, located at least 80% of the bound radioactivity in the lipid extract. That the lipid-free residue cannot be ignored in the search for the dibenamine-receptor complex is apparent from the finding of a significant increment in radioactivity ($P<0·01$) in the lipid-free residue of the control strips, as compared to the lipid-free residue of experimental strips. A similar conclusion regarding the importance of the lipid-free residue was reached by determining the radioactivity in the lipid extract and the lipid-free residue of tissues labeled according to procedure IA. In this case the amount of radioactivity associated

TABLE 3. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE IA

Remaining sensitivity to adrenaline (% of original)	Dry weight of aortic strip (mg)		dis/min in lipid-free residue/mg dry weight of strip		dis/min in lipid extract/mg dry weight of strip		Total dis/min/mg of dry weight of strip	
	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip
62	12·6	13·1	93	127	64	75	157	202
71	13·6	16·3	86	92	38	39	124	131
67	13·0	12·6	83	137	32	56	115	193
50	17·5	14·9	51	84	39	56	90	140
29	15·3	13·7	61	76	48	59	109	135
50	15·1	14·7	78	79	48	49	126	128
70	15·8	13·1	87	79	49	44	136	124
60	13·9	14·0	81	71	55	48	136	119
30	15·8	16·6	63	91	35	53	98	144
Mean ± S.D.	14·7 ± 1·5	14·3 ± 1·3	76 ± 22	93 ± 22	45 ± 10	53 ± 10	121 ± 20	146 ± 28
P Value				<0·05		<0·1		<0·05

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue.

TABLE 4. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE II

Remaining sensitivity to adrenaline (% of original)	Dry weight of aortic strip (mg)		dis/min in lipid-free residue/mg dry weight of strip		dis/min in lipid extract/mg dry weight of strip		dis/minTotal /mg of dry weight of strip	
	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip
30	18·6	18·7	121	83	21	14	142	97
50	12·8	14·8	88	58	27	11	115	69
25	21·2	18·6	101	76	36	18	137	94
15	15·3	16·3	105	106	32	31	137	137
25	21·0	18·4	79	77	36	23	115	100
30	17·0	22·0	111	69	27	19	138	88
25	15·6	15·7	114	91	35	24	148	115
Mean ± S.D.	17·4 ± 2·9	17·8 ± 2·3	103 ± 14	80 ± 14	31 ± 5	20 ± 6	133 ± 12	100 ± 20
P Value			<0·01		<0·005		<0·005	

The total dis/min/mg dry weight of strip is the sum of the dis/min/mg of the lipid extract and lipid-free residue

TABLE 5. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE COMPONENTS OF LIPID EXTRACTS OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELLED BY PROCEDURE IA

R_f value	17·5 μg ^{14}C-dibenamine HCl	Total dis/min per segment of chromatogram							
		Experiment 1		Experiment 2		Experiment 3		Experiment 4	
		Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip
0–0·04	32	0	4	1	0	0	2	0	1
0·04–0·16*	25	4	5	0	0	1	0	0	1
0·16–0·34†	59	3	4	1	1	0	2	0	0
0·34–0·63‡	94	3	3	2	6	0	6	0	0
0·63–0·87	215	2	5	8	7	2	1	2	0
0·87–1	20,173	55	70	37	90	39	39	45	46
Total	20,598	67	91	49	104	42	50	47	48

* Range for sphingomyelin.
† Range for phosphatidyl serine.
‡ Range for cephalin.

TABLE 6. THE DISINTEGRATIONS PER MINUTE DETECTED IN THE COMPONENTS OF LIPID EXTRACTS OF EXPERIMENTAL AND CONTROL RABBIT AORTIC STRIPS LABELED BY PROCEDURE II

R_f value	17·5 μg ^{14}C-dibenamine HCl	Total dis/min per segment of chromatogram							
		Experiment 1		Experiment 2		Experiment 3		Experiment 4	
		Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip	Control strip	Experimental strip
0–0·03	32	0	0	0	0	1	0	0	0
0·03–0·18*	25	8	0	0	0	1	0	4	0
0·18–0·35†	59	2	0	0	1	5	2	5	0
0·35–0·60‡	94	3	4	2	3	4	6	7	6
0·60–0·84	215	3	4	2	2	2	3	6	7
0·84–1·00	20,173	60	40	95	51	101	59	90	58
Total	20,598	76	48	99	57	114	70	112	71

* Range for sphingomyelin.
† Range for phosphatidyl serine.
‡ Range for cephalin.

with the lipid fraction was 37% of the total radioactivity bound to the tissue. Dikstein and Sulman[4] extracted the lipids of aortic strips by boiling the tissue with chloroform:methanol (2:1) for 2 min. In our experiments the lipids of aortic strips were extracted according to the method of Folch *et al.*[8] by homogenizing the tissue twice with chloroform:methanol (2:1) at room temperature. In order to check whether there was incomplete removal of lipid-bound ^{14}C-labeled dibenamine in the present study, the residue which was extracted with chloroform-methanol at room temperature was re-extracted twice with hot chloroform:methanol (2:1). However, examination of this extract by thin-layer chromatography showed that there was no lipid present. Moreover, no radioactivity was detected in this extract. The smaller amount of radioactivity located in the lipid extracts in our experiments as compared to those of Dikstein *et al.*[3, 4] cannot be explained by incomplete extraction.

The distribution of radioactivity in the lipid components of the lipid extracts was studied by means of thin-layer chromatography in order to evaluate the claim of Dikstein *et al.* that cephalin is probably the tissue acceptor for dibenamine, The results of this study are recorded in Tables 5 (procedure IA) and 6 (procedure II). The bulk of the radioactivity was detected in the solvent front, which was the position to which ^{14}C-labeled dibenamine hydrochloride moved in this solvent system. Only minor amounts of radioactivity were detected in the phospholipid fractions, and no significant difference was discerned in the amounts of the radioactivity in the phospholipid fractions of experimental and control strips. This finding is contrary to that of Dikstein and Sulman. The latter workers, using a procedure analogous to procedure IA in this study, reported that the radioactivity in the cephalin fraction of experimental strips was greater than that in control strips. Conversely, they detected more radioactivity in the cephalin fraction of control strips than in experimental strips, by a procedure analogous to procedure II in this study.

In seeking to understand the difference between the results obtained by us and those obtained by Dikstein and Sulman,[4] the following facts are of interest: (1) Dikstein and Sulman have not studied the degree of protection of the α-adrenergic receptor afforded by adrenaline (1×10^{-4}) against dibenamine hydrochloride (1×10^{-5}), the concentrations used in their experiments. In view of the report of Furchgott[6] that no protection or doubtful protection of the α-adrenergic receptor is obtained under these conditions, it is likely that the concentrations of adrenaline and dibenamine hydrochloride used were inappropriate. Moreover, the aortic strips used by Dikstein and Sulman were not subjected to any loading tension. (2) Tritium is able to exchange with the labile hydrogen atoms of the solvent or tissue components, and it is possible that a part of the radioactivity detected in the lipid extracts by these investigators might have been due to exchange of tritium for hydrogen.

Further studies are in progress in our laboratory to characterize the ^{14}C-labeled constituents of the lipid extracts and to study the distribution of radioactivity in the nonlipid components of aortic strips.

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