block vagal degranulation, provides a basis for a hypothesis on adrenergic release of 5-HT from the bowel.

CONCLUSION

The experiments were designed to provide additional information on the effects of vagal stimulation on the granulation of the EC system as a link to the release of 5-HT in the bowel. As evidenced by the data, it was confirmed that peripheral electrical stimulation of the cervical vagus produced a reduction of argentaffin-positive EC granulation. This effect was not blocked by atropinization of the animal. C.V.S. and infusion of norepinephrine but not epinephrine produced a similar degranulation. This effect was blocked by both chemical and immunological sympathectomy. Finally, catecholamine-fluorescent fibers were found to be present in the trunk of the cervical vagus in the guinea pig. In short, the mutual agreement in the morphological, histochemical, and physiological observations tested provides a basis for the hypothesis of the noradrenergic release of 5-HT from EC of the guinea pig small bowel.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 27, 1970, from the *Schools of Dentistry and Pharmacy, Temple University, Philadelphia, PA 19140*

Accepted for publication August 20, 1970.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Washington, D.C. meeting, April 1970.

This investigation was supported by Grant AM-11372-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors express their appreciation to Mrs. N. C. Gulezian for her assistance in preparation of the histologic material.

Topical Mosquito Repellents III: Carboxamide Acetals and Ketals and Related Carbonyl Addition Derivatives

P. TSAKOTELLIS*, H. L. JOHNSON†, W. A. SKINNER†, D. SKIDMORE‡, and H. I. MAIBACH‡

Abstract □ Slow release of topically applied mosquito repellents was attempted *via* formation of low volatility carbonyl addition derivatives of repellent alcohols and carbonyl compounds. Sufficiently rapid hydrolytic release of volatile, repellent moieties on the skin surface did not occur. Vapor repellency was due to the intact derivatives and was found to be dependent upon volatility characteristics. It was concluded that optimum volatility for prolonged topical repellency varies with each homologous series. Compounds incorporating both an acetal and an amine function, although not comparable to diethyltoluamide in topical repellency, appear to be worthy of additional study.

Keyphrases □ Mosquito repellants, topical—synthesis □ Carboxamide acetals, ketals—synthesis □ Repellency, mosquito—volatility relationship

In previous studies of mosquito repellency among structural analogs of diethyltoluamide (DEET), the dominant significance of volatility was demonstrated as a factor in both potency and duration of topical repellent action (1, 2). A limitation is imposed on the concept that duration is maximized by decreased volatility owing to a concomitant decline in potency (1). In the present study an alternative means of prolongation of repellent action through slow release was examined. In addition, the authors examined the effect on repellency of functional group alteration of various repellent carbonyl compounds and extended studies on the relationship between volatility and repellency to classes of compounds other than amides.

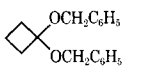
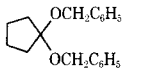
Attempts at slow release were based on the assumption that hydrolysis (chemical or enzymatic) of acetal-type derivatives on the skin surface could result in a prolonged release of the parent alcohol and carbonyl moieties as repellent agents. Choice of repellent moieties was based partly on preliminary estimations of "in-

Table I—Repellency of Intermediates and Standards

Compound	B.p.	Duration of Topical Repellency, hr.[a]
Acetophenone	202°	<0.25
Benzyl alcohol	205°	<0.25
n-Butanol	118°	<0.25
p-Chloroacetophenone	236°	1.0
Cycloheptanone	180°	2.0
Cyclohexanone	156°	1.0
Cyclopentanone	131°	<0.25
N,N-Dimethylacetamide	165°	1.0[b]
N,N-Dimethylformamide	153°	1.0[b]
N,N-Diethyltoluamide	300°	6–10[b]

[a] Time of first confirmed bite; application to human forearm at 2 mg./cm.^{2} except where otherwise noted. [b] Application at 0.35 mg./cm.^{2}.

Table II—Comparative Repellency on Skin and Cloth

Compound[a] Number	Structure	Duration of Repellency, hr.[b] Skin	Cloth
22		5	>4
23		2	8
N,N-Diethyl-toluamide[c]		12–16	—

[a] See Table V. [b] Application to skin and to cheesecloth at 2.0 mg./cm.^{2}; cheesecloth was positioned 0.5–1.0 cm. above skin surface. In all cases, repellency was apparent on initial test (0.25 hr.) and thereafter for indicated duration. [c] Obtained from Eastman Organic Chemicals.

trinsic repellency" of various functional classes. These estimations were made by a rapid screening method comparing olfactometric repellency of compounds of similar volatility on exposure of approximately identical evaporative surface areas to carrier airstreams. Two volatility ranges were examined (b.p. ~100° and ~200°). These preliminary observations indicated that compounds which would hydrolyze to volatile alcohols, aldehydes, ketones, and amides might be suitable compounds for slow-release studies. Topical duration was not of primary concern with regard to intermediates because of intended slow release. In fact, poor duration was expected due to selection of high volatility for optimization of repellent potency (1, 2). This was verified in a few cases by skin testing where only the less volatile compounds tested exhibited repellency more than 15 min. after application (Table I).

In contrast to the poor topical repellency of the alcohol and carbonyl intermediates, somewhat enhanced topical repellency was observed for some derivatives incorporating these relatively volatile moieties (Table II). However, the lack of greatly extended repellency duration or of latent appearance of repellency in tests conducted over periods of several hours suggested that sufficiently rapid hydrolytic release on the skin surface was not occurring with the candidate compounds. This conclusion was substantiated in parallel tests with selected compounds in which the substances were applied both on cloth and directly to skin and the protection times compared (Table II). Since protection was immediate and protection times were comparable in the two methods (or prolonged on cloth), it was concluded that repellency may be due primarily to the intact derivative and not to hydrolysis on the moist, acidic skin surface. The compounds reported herein appear to be relatively stable under the test conditions; all subsequent discussion refers to the intact structures listed in Tables III–VIII.

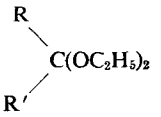
As expected on the basis of previous findings, all of the highly volatile diethyl ketals (Table III) were ineffective topically. This was presumably due to rapid evaporative loss during solvent evaporation (1). Only Compound 3, whose boiling point is comparable to that of DEET, showed significant protection. Similar findings apply to the di-*n*-butyl ketals (Table IV). Furthermore, topical repellency could only be demonstrated with application of very large amounts. Only with the dibenzyl ketals (Table V) was volatility sufficiently low to allow significant durations of repellency at an application rate of 2 mg./cm.^{2}. However, very low volatility resulted in inactive compounds (Compounds 20, 21, 25, and 27) due to insufficient vapor concentration (1). In this series, a volatility corresponding to a boiling point of 150° (0.5 mm.) appears to be approximately optimal.

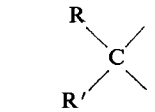
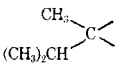
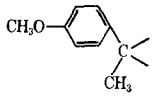
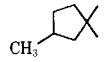
The acetals examined (Table VI) exhibited poor activity at 2.0 mg./cm.^{2} in spite of a seemingly favorable volatility range. On the other hand, the carboxamide acetals and ketals (Table VII) exhibited some repellency characteristics over a wide range of volatility. The orthoesters examined (Table VIII) were ineffective, presumably because of their volatility characteristics.

The present results emphasize the generality of volatility considerations in topical repellent design. Thus, the decline in topical effectiveness with increasing volatility (b.p. <100°, 0.5 mm.) seen previously with various amides (1) is apparent with other compounds as well. Likewise, the loss of effectiveness with decreasing volatility beyond a rather narrow optimal range is apparent, as in the case of the amides (1). However, while the optimum volatility for various amides corresponds approximately to a boiling point of 100° (0.5 mm.) (1), that for the ketals and acetals seems to correspond to approximately a boiling point of 150° (0.5 mm.). Since many factors, such as spreading characteristics, absorption rates, and binding to protein, can affect rates of topical vaporization, this difference is difficult to interpret. It is apparent, however, that optimum volatility characteristics for prolonged topical repellency are different for different classes of compounds and must be determined experimentally for each homologous series.

As a group, acetals and ketals do not represent a particularly promising repellent class. Even optimal members of the series exhibited protection times comparable to amides such as DEET only at concentrations five times greater than those of the latter substances (1). However, the addition of an amine function, as in the case of acetal and ketal derivatives of amides, appears to improve repellency characteristics. Unfortunately, initial attempts to prepare carboxamide acetals and ketals of lower volatility in pure form were unsuccessful, but the class appears worthy of additional study.

Table III—Diethylketals



Compound Number		Yield, %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.^{2})
1		41	$C_9H_{20}O_2$	50–51° (21)[a]	<25°	0.25
2		69	$C_{12}H_{18}O_2$	58–60° (0.45)[b]	59°	1.0
3		70	$C_{13}H_{20}O_3$[c]	77–78° (0.20)	95°	2.0
4		92	$C_9H_{18}O_2$	55–56° (24)[d]	<25°	0.25
5		83	$C_8H_{16}O_2$	140–141°[e]	<25°	0.25
6		88	$C_9H_{18}O_2$	62–64° (20)[f]	<25°	0.25
7		93	$C_{10}H_{20}O_2$[g]	63–64° (12)	<25°	0.25
8		59	$C_9H_{17}ClO_2$	81–83° (12)[h]	35°	0.25
9		65	$C_{10}H_{20}O_2$	30–32° (0.45)[i]	31°	0.25
10		85	$C_{11}H_{22}O_2$	79–81° (13)[i]	31°	0.25
11		94	$C_{11}H_{22}O_2$	125–127° (57)[i]	45°	0.25

[a] Lit. (3) b.p. 52.4° (20 mm.). [b] Lit. (4) b.p. 110–112° (12 mm.). [c] Calcd. for C, H: 69.6, 9.0; Found: 69.7, 9.0. [d] Lit. (5) b.p. 55° (23 mm.). [e] Lit. (6) b.p. 138–141°. [f] Lit. (7) b.p. 63–65° (20 mm.). [g] Calcd. for C, H: 69.7, 11.7; Found: 69.1, 11.7. [h] Lit. (8) b.p. 93–95° (15 mm.). [i] Lit. (9) b.p. 72° (9 mm.) for Compound 9, b.p. 76–77° (15 mm.) for Compound 10, and b.p. 88–89° (15 mm.) for Compound 11.

EXPERIMENTAL[1]

Except where otherwise noted, compounds were purified and boiling points (uncorrected) were determined by vacuum distillation through a 10-cm. Vigreux column.

Diethylketals—All the diethylketals, except that from cyclobuaneone, were prepared from ethanol, triethyl orthoformate, and the corresponding ketone, using a trace of mineral acid as a catalyst. The diethylketal of cyclobutanone (boiling point similar to that of triethyl orthoformate) was prepared from ethanol and the diethylketal of cyclopentanone. The diethylketal of cyclobutanone was only distilled at atmospheric pressure.

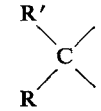
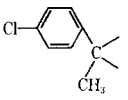
Di-*n*-butylketals—All the di-*n*-butylketals were prepared by the method of MacKenzie and Stocker (10) from *n*-butanol, triethyl orthoformate, and the corresponding ketone, using a catalytic amount of *p*-toluenesulfonic acid. The mixture was slowly heated at such a rate that the ethyl formate removal through a 10-cm. Vigreux column started after 1 hr.; the heating was continued until all the ethyl formate and the ethanol formed were removed. The mixture was cooled, neutralized with a dilute solution of sodium in *n*-butanol, washed with water, dried, and fractionally distilled.

Dibenzylketals and Acetals—These compounds were prepared by transketalization of the corresponding diethylketals and diethylacetals using a trace of mineral acid, according to the method of Lorette and Howard (18). The mixture was heated at 130–140°, and the ethanol was removed from the reaction mixture through a 10-cm. Vigreux column. Two or more moles of benzyl alcohol per mole of diethylketal gave predominately the dibenzylketal with small amounts of the ethylbenzylketal, depending on the completeness of removal of the ethanol. The mixture was cooled, neutralized with a dilute solution of sodium in benzyl alcohol, and fractionally distilled except for the dibenzylketals of acetophenone, *p*-methoxyacetophenone, cyclohexanone, and cycloheptanone which, on distillation, decomposed to the unsaturated ether and benzyl alcohol. Pentane solutions of these compounds were purified by rapid filtration through basic aluminum oxide.

Carboxamide Acetals—These were synthesized (15) by adding dropwise under cooling an excess of thionyl chloride to the corresponding *N,N*-dialkylformamide. After stirring at 20°, the mixture was heated at 40° *in vacuo* to remove sulfur dioxide. The off-white crystals of the corresponding dichloroamine were dissolved in chloroform and were added dropwise to an ice-cold solution of sodium in the corresponding alcohol. The mixture was stirred 3–4 days at 20° or heated. The sodium chloride was removed and the filtrate was fractionally distilled.

[1] Elemental analyses are by the Microanalytical Laboratory, Department of Chemistry, Stanford University, Stanford, Calif.

Table IV—Di-*n*-butylketals

Compound Number		Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
12		59	$C_{11}H_{24}O_2$			71–72° (8)[a]	30°	0.25
13		63	$C_{16}H_{26}O_2$			67–69° (0.2)[a]	80°	0.25
14		75	$C_{16}H_{25}ClO_2$	C, 67.4 H, 8.9 Cl, 12.4	C, 66.9 H, 8.6 Cl, 12.1	113–115° (2.4)	90°	0.25[b]
15		63	$C_{12}H_{24}O_2$	C, 71.9 H, 12.0	C, 71.7 H, 12.1	108–109° (16)	55°	0.25
16		79	$C_{13}H_{26}O_2$	C, 72.8 H, 12.2	C, 72.6 H, 12.1	82–84° (3)	60°	0.25[c]
17		80	$C_{14}H_{28}O_2$			98–100° (5)[a]	65°	0.25
18		80	$C_{15}H_{30}O_2$	C, 74.3 H, 12.5	C, 74.0 H, 12.3	69–70° (0.2)	80°	1.0

[a] Lit. (10) b.p. 64° (2.3 mm.) for Compound 12, b.p. 85° (0.3 mm.) for Compound 13, and b.p. 89° (1.9 mm.) for Compound 17. [b] 4 mg./cm.²; 1.0 hr. [c] 4 mg./cm.²; 0.25 hr.

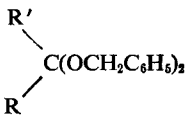
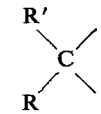
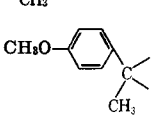
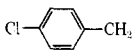


Table V—Dibenzylketals

Compound Number		Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
19		42	$C_{17}H_{20}O_2$			124–126° (0.45)[a]	125°	0.25
20		58	$C_{22}H_{22}O_2$	C, 82.9 H, 7.0	C, 82.7 H, 6.7	dec.[b]	dec.	0.25
21		47	$C_{23}H_{24}O_3$	C, 79.2 H, 6.9	C, 79.5 H, 6.6	dec.[b]	dec.	0.25
22		67	$C_{18}H_{20}O_2$	C, 80.5 H, 7.5	C, 80.0 H, 7.5	124–125° (0.15)	146°	2.5[c] 4.0 5.0
23		61	$C_{19}H_{22}O_2$	C, 80.8 H, 7.9	C, 80.1 H, 8.2	140° (0.20)	150°	5.5[c] 2.0 2.0
24		45	$C_{20}H_{24}O_2$	C, 81.0 H, 8.2	C, 80.9 H, 8.1	140–142° (0.15)	160°	2.0
25		60	$C_{20}H_{24}O_2$	C, 81.0 H, 8.2	C, 80.4 H, 7.6	dec.[b]	dec.	0.25
26		83	$C_{21}H_{26}O_2$	C, 81.2 H, 8.4	C, 81.8 H, 8.0	160–162° (0.07)	190°	1.0
27		71	$C_{21}H_{26}O_2$	C, 81.2 H, 8.4	C, 80.8 H, 8.3	dec.[b]	dec.	0.25

[a] Lit. (11) b.p. 125° (1 mm.). [b] Pentane solution was purified by rapid filtration through basic aluminum oxide. [c] Duration of repellency varies considerably when material is applied at the same levels to different subjects.

Table VI—Acetals $(RCHOR')_2$

Compound Number	R	R′	Yield, %	Formula	Anal., % Calcd.	Anal., % Found	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
28	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	79	$C_{13}H_{28}O_2$			86–88° (2.5)[a]	60°	0.25
29	$CH_3(CH_2)_3$	$C_6H_5CH_2$	61	$C_{19}H_{24}O_2$	C, 80.2 H, 8.5	C, 80.0 H, 8.6	136–138° (0.20)	152°	1.0
30	$CH_3(CH_2)_3$		44	$C_{19}H_{22}Cl_2O_2$[b]	C, 64.4 H, 6.3	C, 64.3 H, 6.3	174–175° (0.30)	180°	0.25
31	$CH_3(CH_2)_3$		58	$C_{19}H_{32}O_2$	C, 78.0 H, 11.0	C, 78.1 H, 10.9	119° (0.30)	130°	0.25
32	$CH_3(CH_2)_3$		49	$C_{21}H_{36}O_2$	C, 78.7 H, 11.3	C, 78.9 H, 11.3	135° (0.15)	155°	0.25
33	$CH_3(CH_2)_3$		60	$C_{23}H_{40}O_2$	C, 79.2 H, 11.5	C, 79.5 H, 11.5	146–148° (0.15)	170°	0.25
34	C_6H_5	$C_6H_5CH_2$	7	$C_{21}H_{20}O_2$			170–171° (0.15)[c]	195°	0.25
35			41	$C_{24}H_{40}O_2$	C, 79.9 H, 11.1	C, 79.5 H, 11.1	150–152° (0.15)	175°	0.25

[a] Lit. (12) b.p. 87° (2.5 mm). [b] Analysis, Cl: calcd., 20.0; found, 20.0. [c] Lit. (13) b.p. 170° (0.15 mm.).

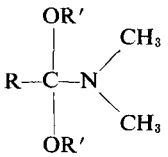


Table VII—Carboxamide Acetals and Ketals

Compound Number	R	R′	Yield,[a] %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
36	H	$CH_3(CH_2)_3$	26	$C_{11}H_{25}NO_2$	101–103° (11)[b]	50°	0.25
37	H	$(CH_3)_3CCH_2$	—[c]	$C_{13}H_{29}NO_2$	85–87° (10)	40°	0.25
38	H		—[c]	$C_{15}H_{29}NO_2$	115–116° (0.75)	110°	1.00
39	C_6H_5	C_2H_5	48	$C_{13}H_{21}NO_2$	57–58° (0.4)[d]	58°	1.00
40	C_6H_5	$CH_3(CH_2)_3$	79	$C_{17}H_{29}NO_2$[e]	86–87° (0.15)	100°	1.00

[a] Yields were calculated as to the starting amide. [b] Lit. (14) b.p. 96–98° (16 mm.). [c] Compounds were obtained from Aldrich Chemical Company. [d] Lit. (15) b.p. 60° (0.5 mm.). [e] Calcd. for C, H, N: C, 73.0, H, 10.4, N, 5.0; Found: C, 72.9, H, 10.4, N, 5.0.

Carboxamide Ketals—Dry phosgene was bubbled through an ice-cold benzene solution of *N,N*-dimethylbenzamide (15). During the addition, white crystals precipitated; the mixture was stirred 16 hr. at 20° and then was evaporated to dryness. The hygroscopic dichloramine was dissolved in chloroform and was added dropwise to an ice-cold solution of sodium in the corresponding alcohol. The mixture was stirred 2 days at 20° and was worked up as for the carboxamide acetals.

Orthoesters—Tribenzyl orthoformate was prepared from triethyl orthoformate by removal of the ethanol formed, according to the method of Alexander and Buch (16). Triethyl orthobutyrate was prepared by alcoholysis of 1,1-dichlorobutyldiethylamine, which was prepared from the *N,N*-diethylbutyramide and phosgene.

Biological Evaluation—Repellency duration testing was conducted by uniform application of repellent in EtOH to an exposed area of the arm of a human subject as described previously (1). Female *Aedes aegypti* (nonblood fed) mosquitoes (5–7 days old) were used in all tests.

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Table VIII—Orthoesters

Compound Number	Structure	Yield, %	Formula	B.p. (mm.)	B.p. (0.5 mm.)	Repellency, hr. (2.0 mg./cm.²)
41	$HC(OCH_2CH_2CH_2CH_3)_3$	—[a]	$C_{13}H_{28}O_3$	75° (0.5)	75°	0.25
42	$HC(OCH_2C_6H_5)_3$	72	$C_{22}H_{22}O_3$	dec.[b]	dec.	0.25
43	$CH_3(CH_2)_2C(OC_2H_5)_3$	69	$C_{10}H_{22}O_3$	62–63° (10)[c]	<25°	0.25

[a] The compound was obtained from Eastman Organic Chemicals Co. [b] Lit. (16). [c] Lit. (17) b.p. 58–59° (7 mm.).

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 12, 1970, from the *Department of Pharmaceutical Chemistry, Life Sciences, Stanford Research Institute, Menlo Park, CA 94025*

Accepted for publication September 1, 1970.

This work was supported by the U. S. Army Medical Research and Development Command Contracts DA-40-193-MD-2466 and DA-49-193-MD-2465.

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In Vitro Binding Study of Epinephrine and Bovine Serum Albumin

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Abstract □ The binding of epinephrine to bovine serum albumin and to acetylated bovine serum albumin was studied with respect to evaluation of the active site on the small molecule and to the exchange rate. The effects of varying concentrations of bovine serum albumin on the relaxation rate and chemical shift of 0.1 M (molal) epinephrine were examined. Temperature and pH effects on the relaxation rate of various epinephrine lines were examined. A comparison of relaxation rates between epinephrine–bovine serum albumin and epinephrine–acetylated bovine serum albumin was made. NMR instrumentation was used to follow the reactions. It was found that binding between epinephrine and bovine serum albumin was detectable with this instrumentation and that the most probable active site on the small molecule was the alkyl side chain. It also appeared that there was a fast exchange rate between bound and unbound epinephrine.

Keyphrases □ Epinephrine binding—bovine serum albumin □ Bovine serum albumin, acetylated bovine serum albumin—epinephrine binding □ Temperature, pH effects—epinephrine–bovine serum albumin relaxation rates □ NMR spectroscopy—analysis

The interaction of hormones with plasma proteins is attracting increasing interest because of the involvement of binding in events that are of considerable physiological and pharmacological importance. The numerous studies in this area have been the subjects of several extensive review articles (1–4). During the last decade, there have been a number of conflicting reports concerning whether or not the endogenous hormones, epinephrine and norepinephrine, are bound to plasma proteins. In 1958, Antoniades *et al.* (5), employing dialysis, cationic exchange-resin techniques, and chemical and biological assays, concluded that although epinephrine was completely bound to plasma proteins an appreciable amount of norepinephrine seemed to be unbound, and that the plasma protein responsible for the binding and transport of these hormones was albumin.

In 1962, Bickel and Bovet (6) utilized the method of "crossing paper electrophoresis" and postulated that there was no interaction between these hormones and dog serum albumin. However, during the same year, Litt (7) published the results of his equilibrium dialysis studies and indicated that there was binding between epinephrine and bovine serum albumin (BSA).

In 1968, Cohen *et al.* (8), using paper electrophoresis, showed that isotopically labeled epinephrine and norepinephrine were bound by rabbit serum albumin and also by α- and β-globulin. Neither this nor any of the previous studies has defined the active site of binding on the adrenergic molecules.

Recent studies have established the value of NMR spectroscopy as a tool for conformational determinations of pharmacologically active molecules in solution (9–11), for the elucidation of interactions between small molecules (10, 11), for studying protein small molecule interactions, and in assessing the extent to which various functional groups on the small molecule participate in the interaction (12–16).

In the study reported here, NMR spectroscopy was used to investigate the binding of epinephrine to BSA and to acetylated BSA. The results of this study suggest that epinephrine is bound to BSA and that the active site for binding is located on the aliphatic side chain of epinephrine.