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Inhibition of the Release of Slow-Reacting Substance of Anaphylaxis in the Rat with Diethylcarbamazine* (32638)

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Diethylcarbamazine citrate (Hetrazan) is an effective chemotherapeutic agent against microfilarial infestations in animals and man (1); however, the mode of action of this piperazine derivative on microfilariae remains to be established. Hetrazan has also been utilized with success in the treatment of tropical eosinophilia when no microfilarial infestation could be demonstrated (2). More recently, Mallén noted the efficacy of this drug in the relief of the bronchospastic symptoms associated with tropical eosinophilia, and this observation prompted him to perform a brief clinical trial with Hetrazan in "intractable asthma" (3). Fourteen of 15 patients responded satisfactorily to Hetrazan with only minimal side effects.

Slow-reacting substance of anaphylaxis (SRS-A) has been suggested as a chemical mediator involved in the pathogenesis of human asthma (4). Brocklehurst (5) has demonstrated the release of SRS-A_{hu} from the perfused segments of lung of 2 pollen-sensitive individuals and from a bronchial ring preparation which contracted in response to specific antigen. In addition, human bronchiolar tissue is the only bronchiolar tissue known to be very sensitive to guinea pig SRS-A_{gp} (4).

The immunologic release of SRS-A_{rat} has been accomplished *in vivo* in the rat using both heterologous (6) and homologous (7) antisera. The SRS-A_{rat} recovered from the rat peritoneal cavity was pharmacologically in-

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distinguishable from the SRS-A_{gp} recovered from perfused, shocked guinea pig lungs (6). The present study describes the inhibition of the antigen-induced release of SRS-A_{rat} with Hetrazan.

Materials and Methods. Male Sprague-Dawley rats weighing 200–300 gm were passively sensitized by the intraperitoneal administration of 1.0 ml of rabbit hyperimmune antiserum to bovine serum albumin (BSA) (Pentex) containing 3–11 mg/ml of specific antibody protein. Four hours later, the animals were challenged with 2.0 mg BSA in 1.0 ml of 0.15 *M* saline intravenously, followed immediately by the intraperitoneal injection of 5.0 ml of Tyrode's solution containing heparin, 50 μg/ml. Exactly 5 min later, the animals were stunned and exsanguinated from the carotid artery. The abdominal wall was incised and reflected; the peritoneal fluid was aspirated with siliconized Pasteur pipettes and collected in iced centrifuge tubes. The fluid was centrifuged at 150*g* for 4 min at 4°C, and the supernatants were collected in iced polypropylene tubes. The cell buttons were resuspended in 3.0 ml of Tyrode's solution and boiled for 8 min to extract the residual cellular histamine.

Histamine and SRS-A_{rat} were assayed on the isolated guinea pig ileum as described in (7). One unit of SRS-A_{rat} was arbitrarily defined as the concentration required to produce a contraction with an amplitude equivalent to that obtained with 5 mμg of histamine base in that assay. Representative samples were also assayed for serotonin and bradykinin using the estrous rat uterus (7). Insignificant concentrations of these mediators were found.

Hetrazan (diethylcarbamazine citrate, Lederle) in 0.15 *M* saline was administered to rats intravenously at the time of challenge with specific antigen and at varying times before challenge. The dosage ranged from 5–20 mg/kg, and the rats tolerated these doses with no obvious detriment to their well being.

Passive cutaneous anaphylaxis (PCA) was performed in male Sprague-Dawley rats weighing 200-350 gm (8). Hyperimmune rabbit antisera of various dilutions (0.1 ml each) were injected intradermally into the shaved dorsal skin of rats, and no more than 15 sites were injected in each animal. Four hours later, the rats were injected intravenously with 1.0 ml of a solution containing 1.0 mg of specific antigen in 0.5% Evan's blue dye (Allied). Forty-five minutes after the injection of antigen, the rats were killed in ether; the reflected skin was transilluminated and examined for the intensity of blueing and the diameters of the lesion.

White blood cell counts, cell differentials, and whole serum complement determinations (9) were carried out on normal rats and rats pretreated with Hetrazan, 20 mg/kg iv, 5 min before exsanguination. Precipitin analyses (10) with rabbit hyperimmune antisera were carried out in the presence of 20 m*M* Hetrazan and compared with those performed in 0.15 *M* saline alone.

Results. Hetrazan inhibition in the bioassay system. The effect of Hetrazan on the bioassay system was ascertained. Using histamine in concentrations ranging from 10 to 500 mμg histamine base, it was established that a ratio of histamine (mμg) to Hetrazan (1×10^{-3} *M*) of 10:1 was required to produce 100% inhibition of the expected contraction. For example, 10×10^{-3} *M* Hetrazan blocks 100 mμg histamine base in contrast to 10^{-6} *M* mepyramine maleate (Neo-Antergan, Merck, Sharp and Dohme) which blocks 200 mμg histamine base. Thus, Hetrazan is a weak antihistamine.

It was determined that 10 m*M* Hetrazan 2.5 mg/ml) blocked 0.5 units of SRS-A_{rat} but had no effect on 1.0 unit; 5 m*M* Hetrazan had no effect on the assay of SRS-A_{rat} as determined in the range of 0.5–4.0 units. Since the highest dose of Hetrazan used in these experiments was 20 mg/kg and the volume of peritoneal fluid harvested about 4–5 ml, the maximum concentration of Hetrazan possible in the peritoneal fluid was about 1.0 mg/ml. It should be noted that the concentration of SRS-A_{rat} in the peritoneal fluid of rats not receiving Hetrazan was never less than 50 units/ml. Thus, neither the concentration of Hetrazan used nor the quantity of SRS-A_{rat} released from control animals is in a range such that Hetrazan could influence the bioassay.

Effect of Hetrazan on the antigen-induced release of SRS-A_{rat}. Initial experiments con-

TABLE I. Hetrazan Inhibition of SRS-A_{rat} Release.[a]

Experiment	SRS-A_{rat} release (units)		Inhibition (%)
	Control	Treated	
A	170	17	90
B	385	38	92

[a] Each result represents the mean value for 3 rats sensitized with 1.0 ml of rabbit anti-BSA containing (A) 3.7 mg and (B) 11.4 mg of specific antibody protein/ml and challenged 4 hours later with 2.0 mg BSA iv in 1.0 ml 0.15 *M* saline. Hetrazan, 20 mg/kg, administered simultaneously with the antigen to the treated groups.

cerned with Hetrazan inhibition of *in vivo* SRS-A_{rat} release were carried out with a drug dosage of 20 mg/kg iv at the time of challenge with specific antigen. Two such experiments are depicted in Table I. The Hetrazan treated groups in both experiments demonstrated at least 90% suppression of SRS-A_{rat} release. The dose-response to Hetrazan, expressed as percentage inhibition of SRS-A_{rat} release, is described in Table II. In this experiment, Hetrazan in a dose of 5 mg/kg produced 25% inhibition of SRS-A_{rat} release whereas 20 mg/kg gave 71% inhibition.

In separate experiments, passively sensitized rats were treated with Hetrazan, 20 mg/kg iv, 0, 5, 15, 60, and 240 min before challenge with specific antigen. The results of these experiments are documented in Table III. The percentage inhibition of each of these treated groups is expressed by comparison with the mean SRS-A_{rat} release of an untreated group in that experiment. No significant suppression of SRS-A_{rat} release is observed when this dose of Hetrazan is administered 15 min or longer before antigen challenge.

TABLE II. Dose-Response of Hetrazan Inhibition of SRS-A_{rat} Release.[a]

Hetrazan (mg/kg)	SRS-A_{rat} release	Inhibition (%)
—	197	—
5	148	25
10	117	41
20	58	71

[a] Each figure represents the mean result for 3 rats sensitized with 1.0 ml of rabbit anti-BSA (5.7 mg specific antibody protein/ml) and challenged 4 hours later with 2.0 mg BSA in 1.0 ml 0.15 *M* saline iv. Hetrazan and antigen administered simultaneously in the same syringe.

Passive cutaneous anaphylaxis. Heterologous, hyperimmune antiserum which is capable of preparing the rat peritoneal cavity for the antigen-induced release of SRS-A_{rat} is also capable of preparing rat skin for PCA after a 4-hour but not a 48-hour latent period (7). Histamine and serotonin antagonists have not abolished this reaction (11), whereas these agents have prevented the 48-hour PCA reaction produced by homologous, "mast cell sensitizing" (homocytotropic) antibody (11), and the blueing response following the intradermal injection of rabbit anti-rat mast cell antiserum (12, 13). An experiment was therefore carried out comparing the effects of mepyramine maleate and methysergide, Hetrazan, and a combination of the three on the 4-hour PCA reaction with heterologous, hyperimmune antiserum and the blueing reaction mediated by rabbit anti-rat mast cell antiserum. It is evident from Table IV that mepyramine maleate and methysergide blocked the capacity of the rabbit anti-rat mast cell antiserum to elicit blueing whereas Hetrazan had no effect on this reaction. The 4-hour PCA reaction was partially blocked by

TABLE III. Pretreatment of Rats with Hetrazan at Different Intervals Before Challenge with Antigen.[a]

	Interval (min)	SRS-A_{rat} release		Inhibition SRS-A_{rat} release (%)
		Control	Treated	
A	0	170	17	90
B	5	296	82	72
B	15	296	246	17
C	60	215	265	0
D	240	189	165	12

[a] Four separate experiments are described. Each figure represents the mean result for 3 rats prepared with 1.0 ml of rabbit anti-BSA containing (A) 3.4 mg, (B) 6.3 mg, (C) 11.4 mg, and (D) 3.7 mg of specific antibody protein/ml. Four hours later, rats challenged with 2.0 mg BSA iv in 1.0 ml 0.15 *M* saline. Hetrazan, 20 mg/kg iv, administered at different intervals before challenge.

TABLE IV. Suppression of the 4-Hour PCA Reaction and the Response to Rabbit Anti-Rat Mast Cell Antiserum (Ra anti-RMC) in the Rat.[a]

	PCA								Ra anti-RMC			
	Dilutions of rabbit antiserum								Dilutions of Ra anti-RMC			
Inhibitors	1:1		1:3		1:9		1:27		1:50		1:100	
—	3+	20	3+	14.5	2+	6.0	1+	3.0	4+	16.5	4+	18
Mepyramine maleate + methysergide	4+	15	3+	11.3	1+	4.5	0	0	0	0	0	0
Hetrazan	3+	19.5	3+	15	2+	7.5	1+	4.5	3+	15	3+	14.5
Mepyramine maleate, methysergide, Hetrazan	1+	3.7	Tr	3.3	Tr	2.0	0	0	Tr	3.0	Tr	3.0

[a] Rabbit anti-BSA, 5.7 mg/ml of specific antibody protein, injected intradermally in various dilutions into the dorsal skin of rats. Four hours later, the same rats received intradermal injections of 2 dilutions of Ra anti-RMC. Immediately thereafter, the rats were challenged with 1.0 mg BSA in 0.5% Evan's blue dye iv. Intensity (0–4+) and diameter (mm) of reactions expressed as the mean value for 3 rats. Mepyramine maleate, 50 mg/kg ip, and methysergide (UML 491, Sandoz Pharmaceuticals) 4 mg/kg iv, administered 30 min before challenge. Hetrazan, 20 mg/kg iv, administered at the time of antigen challenge.

mepyramine maleate and methysergide while Hetrazan alone had no effect; pretreatment with all three inhibitors produced virtually complete suppression.

Discussion. Hetrazan appears to inhibit the immunologic release of SRS-A_{rat} *in vivo* in the rat. The maximum dose of Hetrazan used in these experiments (20 mg/kg iv) is well below the LD_{50} for rats which is about 150 mg/kg iv (14). The mechanism of action of this drug on the reaction sequence leading to the formation and release of SRS-A_{rat} has not been defined. Hetrazan is a very weak antihistamine and has a negligible effect on the response to SRS-A_{rat} in the bioassay system. Thus, the inhibition observed can be attributed to some action which prevents the antigen-induced release of SRS-A_{rat} under the experimental conditions described.

A possible mechanism through which Hetrazan might exert a nonspecific inhibitory action on the antigen-induced release of SRS-A_{rat} would be by interference with effective antibody–antigen interaction. However, precipitin analyses of rabbit anti-BSA carried out in duplicate reveal that the presence of Hetrazan in a 20 m*M* concentration had no effect on this type of antibody–antigen interaction. The precipitin curves were superimposable, and at equivalence the antibody titers were 11.1 and 10.7 mg antibody protein/ml in the presence and absence of Hetrazan, respectively.

Since the administration of Hetrazan 15–60 min before antigen challenge does not have an inhibitory effect on SRS-A_{rat} release, it follows that the drug does not act in an irreversible manner and must be present at the time of antibody–antigen interaction in order to prevent release of this mediator. The observation that Hetrazan in a dose of 20 mg/kg is only inhibitory when administered 5 min before or simultaneously with antigen challenge is entirely consistent with the published toxicity studies indicating that the rat is capable of excreting Hetrazan at a rate of 100 mg per kg per hour (14).

Since the polymorphonuclear leukocyte is required for optimal SRS-A_{rat} release(15), the effect of Hetrazan on the number and type of circulating white cells was determined. No appreciable changes were noted (Table V).

The administration of cobra venom (*Naja haje*) is also known to suppress the release of SRS-A_{rat} (15). Whether this action is through a direct effect on the complement system or through some unrelated activity is not known. It was established that Hetra-

TABLE V. Effect of Hetrazan on the CH_{50}, Total, and Differential White Cell Counts of the Rat.[a]

Group	CH_{50} (units/ml)	WBC			
		Total (per mm³)	Differential		
			Poly	Lymph	Mono
Control	165	14,100	23	70	7
Hetrazan	160	12,400	24	69	7

[a] Each result represents the mean value for the 6 rats in each group. Hetrazan (20 mg/kg iv) administered 5 min before the rats were exsanguinated.

zan had no effect on the whole complement activity of the rat (Table V).

The finding that the combined action of Hetrazan with an antihistamine and an antiserotonin agent is needed to fully suppress the 4-hour PCA reaction in the rat mediated by heterologous antibody introduces the possibility that SRS-A_{rat} is one of the chemical mediators of inflammation involved in this reaction. This reaction is clearly distinguished from the PCA reaction elicited with homocytotropic antibody in that the latter involves mast cells, has a prolonged latent period for sensitization, and is completely inhibited by a combination of antihistamine and antiserotonin agents (7,11). Since the intraperitoneal administration of rabbit antisera followed, after an appropriate interval, by challenge with specific antigen leads to the release of SRS-A_{rat} without the concomitant appearance of histamine and serotonin, it is not clear why histamine and serotonin antagonists are required along with Hetrazan for complete suppression of the PCA reaction mediated by heterologous antisera. The polymorphonuclear leukocyte is required for the expression of the heterologous PCA lesion (16) and has recently been shown to be a prerequisite for the *in vivo* release of SRS-A_{rat} (15). Janoff *et al* (17) have demonstrated that polymorphonuclear leukocyte lysosomes contain a fraction which possesses mast cell lytic activity. It is therefore possible that the reaction of antigen with heterologous antibody in rat skin leads to the local formation of SRS_{rat}, a process in which the polymorphonuclear leukocyte is required; and that the participation of this leukocyte in this reaction may result in the release of the mast cell lytic fraction, with the secondary, local release of histamine and serotonin. It is of interest that Lovett *et al.* (16) have described the degranulation and disruption of the mast cells in rat skin during this type of PCA reaction.

Summary. The immunologic release of SRS-A_{rat} with intravenously administered antigen is described. Hetrazan effectively inhibits the antigen-induced release of SRS-A_{rat} in a dose of 20 mg/kg iv. In this concentration, it has no influence on the bioassay system and does not prevent effective antibody–antigen interaction. Hetrazan does not produce an irreversible inhibition and must be present at the time of antibody–antigen interaction to effectively prevent the formation and release of SRS-A_{rat}. Suppression of the 4-hour PCA reaction in the rat with Hetrazan plus mepyramine maleate and methysergide suggests that SRS-A_{rat} may be a permeability factor involved in this reaction.

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Effect of *N*-Cyclohexyl Linoleamide on Cholesterol Metabolism in Rats* (32639)

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The cyclohexylamine amide of linoleic acid (Clinolamide; Linolexamide) has been found to lower serum and liver cholesterol levels and to reduce the severity of atherosclerosis in cholesterol-fed rabbits (1,2). The hypocholesteremic effect of this compound has been attributed in part to inhibition of cholesterol absorption (3), but there are no other published reports concerning its effects on other aspects of cholesterol metabolism. We have investigated the effect of *N*-cyclohexyl linoleamide upon cholesterol metabolism in normocholesteremic rats fed a cholesterol-free semisynthetic diet. The results of this experimental series are the basis of this report.

Methods and Materials. All rats used were males of the Wistar strain. Groups of equal starting weights were used for each experiment: Average starting weights in the biosynthesis experiments were 171 gm, 200 gm, 174 gm, and 160 gm, in experiments 1, 2, 3, and 4, respectively; in the cholesterol oxidation experiment, average starting weights were 171 gm. The rats were maintained for 3 weeks on a diet consisting of mixed cereal, 70 (Pablum, Mead Johnson Co.); wheat germ, 7; skim milk powder, 22; and vitamin mix, 1. The test compound (0.3 or 0.6%) was added to the diet at the expense of the cereal. This diet has been used in other feeding experiments involving drugs and is readily accepted by the rats (4,5).

At the end of the feeding period, the animals were killed by exsanguination. In the cholesterol biosynthesis experiments, liver slices (0.5 gm) were incubated for 3 hours under 100% oxygen in 5 ml phosphate buffer (pH 7), containing 0.006 *M* $MgCl_2$, 0.03 *M* nicotinamide and either 1 μC of sodium acetate-1-^{14}C or 0.5 μC of mevalonic acid-2-^{14}C. In one series, 1.5×10^{-5} moles (10 mg) of *N*-cyclohexyl linoleamide in 0.1 ml of ethanol was added to 0.5 gm of normal rat liver in order to test the *in vitro* effect of this compound on cholesterol biosynthesis. The reaction was stopped by the addition of 5 ml of alcoholic KOH; the sterol was then extracted with petroleum ether and isolated as the digitonide for radioactive assay by liquid scintillation spectrometry (6).

In several experiments the basic solution remaining after extraction of the nonsaponifiable material was acidified and the fatty acids extracted with ether. The ether extracts were dried over anhydrous Na_2SO_4 and an aliquot taken for radioactive assay. A 1-gm aliquot of each liver was taken for cholesterol analysis. In 2 experiments 1 kidney and 1

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