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ARMY REPORT - 1957

The studies contained in this report are extensions of the general theory regarding the function of acidic mucopolysaccharides in the ground substance of the loose connective tissue - that is, as ion exchangers. It is of fundamental interest that the fibroblast is both the "synthesizer" and the catabolizer" of these substances and can thus regulate their qualitative and quantitative distributions in the tissues. On the other hand, these substances are characteristic components of the ground substance which is the sole environment of the fibroblast and can therefore modify the functions of this cell. This interaction of cell and environment constitutes the basis of a regulatory system of the tissues. The distribution of this tissue throughout the body as a packing material of the blood vessels makes this proposed regulatory mechanism of major importance to the physiologic state of the body as a whole. The role of hormones as regulators of the loose connective tissue physiology could very well be thru modification of the ground substance constituents either directly or indirectly via the cell.

The following material contains results of representative studies concerning the roles of the mucopolysaccharides as substances actively participating in the maintenance of the physiologic state of the tissues. Various substances of therapeutic nature such as ACTH, neomycin, polymyxin B have been studied.

TABLE OF CONTENTS

I. Histamine binding and histamine release.

Table 1
Relative Histamine Binding Capacity of Acidic Mucopolysaccharides.

Table 2
Approximate amounts of histamine released from a histamine polysaccharide complex (50 ug histamine + 1000 ug polysaccharide) by 500 ug of 48/80.

II. Acidic mucopolysaccharides and tolerance.

Table 3
Comparison of the Effects of various Acidic Mucopolysaccharides on the Toxicity of Certain Noxious Agents in Mice.

III. Heparin-enhanced therapeutic value of antibiotics.

IV. Influence of ACTH on stress phenomena.

Table 4
Comparison of Various Drugs for Effect on Polymyxin Tolerance and for Dye Release Activity.

Table 5
Effects of ACTH and Other Basic Drugs on Tolerance of Mice to *Polymyxin B*.

V. Factors influencing the toxicity of a snake venom, etc.

Table 6
Effect of Toluidine Blue and Chlorpromazine on Tolerance to Sublethal Doses of Various Toxic Drugs.

Table 7
Effect of Natural Tissue Amines on Histamine Toxicity

VI. Fate of administered heparin and x-irradiation studies.

VII. Steroid sulfates.

VIII. Sulfated macromolecules.

IX. Nucleic Acids.

Discussion

I. Histamine binding and histamine release. Preliminary work (1956 report) indicated that heparin would complex with histamine in a 37% solution of ethanol. This study was expanded to include other mucopolysaccharides and the tests were carried out in a dialysis equilibrium system. Graded concentrations of mucopolysaccharides constituted the inner phase and a standard concentration of histamine was contained in the outer. Volumes were kept standard and systems allowed to equilibrate for 24 hours at room temperature. The binding of histamine by heparin could be measured indirectly by the amount of histamine lost from external phase. Histamine measurements were carried out by dinitrobenzene technique. The relative histamine binding capacities of these acidic mucopolysaccharides are presented in table 1 using neutral dextran as the control.

Table I

Relative Histamine Binding Capacity of Acidic Mucopolysaccharides

Polysaccharide	mg. histamine per mg. mucopolysaccharide*
Heparin	0.94
Heparitin Monosulfate	0.50
Chondroitin Sulfate A	0.60
Chondroitin Sulfate B	0.54
Dextran	0.00

* Free histamine measured by the fluorodinitrobenzene method.

It is shown that various ground substance mucopolysaccharides have a potential for binding histamine and that heparin is the most active in this regard. Other studies were concerned with the dissociation of these histamine-mucopolysaccharide complexes. It was found that either of the in vivo histamine releasers, compound 48/80 or polymyxin B, could effect this dissociation as they concomitantly formed complexes with heparin. This reaction is an ion exchange reaction in which heparin serves as an exchanger. Results of a number of these studies have been used to

compare the relative amounts of histamine released by these histamine releaser (replacer) drugs from standard preparations of histamine-mucopolysaccharide complexes. In table 2 it is shown that 50 ugm histamine is less readily replaced by 500 ugm of compound 48/80 when bound in a complex by heparin than when in complex with an equivalent amount of any of the other test mucopolysaccharides.

Table 2

Approximate amounts of histamine released from a histamine polysaccharide complex (50 ug histamine + 1000 ug polysaccharide) by 500 ug of 48/80

Mucopolysaccharide	ug Histamine Released
Heparin	5
Heparitin Monosulfate	15
Chondroitin Sulfate B	26
Chondroitin Sulfate A	29

Free histamine measured by the fluorodinitrobenzene method.

Under conditions of this test the mucopolysaccharides contained less than half their histamine capacity and other results indicate that with larger amounts of bound histamine, larger amounts of this substance will be released by the same amount of releaser substance. Thus histamine release is related to both the histamine capacity of the mucopolysaccharide as well as the amount of histamine bound. Heparin can therefore be doubly effective in modifying histamine levels since it has the greatest capacity to bind histamine and with a standard amount of bound histamine, releases less in the presence of standard histamine releaser drugs than do other acidic mucopolysaccharides. It should be noted that this relationship of mucopolysaccharides is quite similar to that found for their relative protective effects against lethal intoxication of mice with various noxious agents (see table 3).

II. Acidic mucopolysaccharides and tolerance. Acidic mucopolysaccharides of the ground substance can form acid-base complexes with a variety of noxious cationic

substances. A qualitative and quantitative comparison of the respective abilities of different acidic mucopolysaccharides to maintain these complexes in vivo are shown in table 3. Groups of mice were injected intravenously with single doses of graded amounts of one of the test substances, each dose containing a standard lethal amount of one of the cationic challenge substances. The numbers of surviving animals in each of the challenged was recorded and the amount of mucopolysaccharide necessary to protect 50% of the mice was determined. This procedure was repeated with each of the mucopolysaccharides versus each of the lethal challenge agents.

Table 3

Comparison of the Effects of various Acidic Mucopolysaccharides on the Toxicity of Certain Noxious Agents in Mice

Drugs and Challenge Doses	Mucopolysaccharides			
	Heparin	Heparitin monosulphate	Chondroitin Sulphate A	Chondroitin Sulphate B
	50% Protective Doses (ug/gm)			
1. Compound 48/80 (2.6 ug/gm)	0.36 (±0.015)	12.9 (± 2.97)	none	none
2. Polymyxin B (5.0 ug/gm)	0.56 (±0.005)	5.3 (± 0.875	none	5.4 (±1.575)
3. Clupein (50.0 ug/gm)	7.13 (±0.085)	18.7 (± 4.35)	19.0 (±4.0)	15.35 (±1.35)

* Groups of mice, 10 animals in each, were injected intravenously with single doses of graded amounts of one of the test mucopolysaccharides. Each dose contained a standard lethal amount of the respective challenge agent. Results are presented as the 50% protective dose of mucopolysaccharide with its standard error.

It can be seen that heparin is the most effective inhibitor of the lethal effects of the phenylalkylamine, compound 48/80, the cyclic polypeptide, polymyxin B and the protamine substance, clupein. The relative protective effects can be summarized: heparin $\rangle$ heparitin monosulfate $\rangle$ chondroitin sulfate B $\rangle$ chondroitin sulfate A.

All of these acidic mucopolysaccharides are ingested and digested by fibro-

blasts of the loose connective tissue which also ingest and digest complexes of heparin formed with the above noxious substances. The ground substance mucopolysaccharides can thus serve to reduce the toxic effects of chemically diverse noxious substances and their relative abilities to do so vary both quantitatively and qualitatively. This phenomenon as associated with the fibroblasts of the loose connective tissue has been described as micellophagosis and was the subject of the last report (1956).

III. Heparin-enhanced therapeutic value of antibiotics. As noted in the previous report (1956) and in this year's application, heparin can enhance the therapeutic value of a number of recognized antibiotic substances. Dr. David Dolowitz of this city has been recently cooperating with us on this subject and has found that a number of cases of infected weeping exzemas of the ear which had proven resistant to all types of previous therapy during a course of at least 2 years, could be successfully treated with a combination of heparin and an otherwise excessively high level of appropriate antibiotics (polymycin B, neomycin, streptomycin). The theoretical basis for this use of heparin has considered that it can serve as a biologic repository for various cationic antibiotics in such a manner that the active dose level of the antibiotic can be controlled and thereby prevent injury from high levels of the antibiotic itself. Additionally, heparin can possibly serve as "binder" of various noxious substances produced by the pathogenic microorganisms, such as amines, etc..

Studies in progress have been concerned with screening various toxic antibiotics for possible development as more usable agents. The toxicity of Netropsin, a product of Chas. Pfizer and Co., has already been found to be significantly less in heparinized mice or when administered as a heparin complex. A few other antibiotic agents have also been found to be less toxic under these conditions. A more adequate analysis of these studies will be presented in the next report.

IV. Influence of ACTH on stress phenomena. As shown in the previous studies, the acidic nature of ground substance mucopolysaccharides can govern the tolerance of the tissues to various cationic substances. Non-toxic cationic substances should therefore be able to neutralize their activity in this respect by competing with the toxic agents for the anionic groups of the mucopolysaccharides. A number of basic substances exist in the tissues, some of which are released during stress such as histamine and ACTH. The study was carried out to determine the effect of increasing amounts of ACTH on the tolerance of mice to the histamine releaser, polymyxin B. Groups of mice (10 animals in each) were injected intravenously with single doses of graded amounts of ACTH, each containing a standard sub-lethal amount of polymyxin B. Mice receiving the control dose of polymyxin (3.5 ug/gm) had a 100% survival, plus 5 to 10 ugm ACTH/gm: 80% survival, 25 ugm ACTH/gm 40% survival, 37.5 ug ACTH/gm: 10% survival and 50 ugm ACTH/gm: zero % survival. Deaths occurred within 5 minutes after injection. These results indicating that polymyxin tolerance is reduced proportionate to ACTH dose suggest that ACTH can interfere with a natural mechanism in the tissues for dealing with this substance. Thus an otherwise lethal dose of polymyxin can become equivalent to a lethal dose. Since it has already been shown that heparin and other acidic mucopolysaccharides of the ground substance can enhance tolerance for polymycin, this action of ACTH may be considered as a functional depletion of these acidic mucopolysaccharides.

The importance of ACTH as a hormone made it imperative that further experiments be performed to determine the specificity involved in the ACTH effect on tolerance to polymyxin. Accordingly, a number of other substances were selected for comparison with ACTH. Groups of mice were treated as before and the standard polymyxin dose was given as a mixture with different amounts (25.0, 12.5 or 6.25 ug/g) of the test substance and their effects on the survival ratios were noted. In an associated study, the effect of each of the test drugs on the dissociation of the toluidine blue-heparin complex was also determined. The amount of test substance required

to release 50% (60 ug) of the dye from its heparin complex was designated as the 50% releaser activity of the drug. A substance was arbitrarily considered as ineffective if more than 1 mg of it was required for 50% releaser activity. Comparisons of the various drugs on the basis of their effects on survival ratio and their releaser activities can be observed in table 4.

Table 4

Comparison of Various Drugs for Effect on Polymyxin Tolerance and for Dye Release Activity

Treatment and survival ratios				50% dye release activity (ug)
	Drug doses (ug/g)			
Group	25	12.5	6.25	
		ACTH		150
Control	10/10			
Pmx*	6/15			
		STH		Ineffective
Control	10/10			
Pmx*	"			
		TSH		"
Control	"			
Pmx*	"			
		Chymotrypsin		"
Control	"			
Pmx*	"			
		Clupein		34.5
Control	"			
Pmx*	1/10	3/10	10/10	
		Neomycin		20
Control	0/10	10/10		
Pmx*		0/10	7/10	
		Toluidine blue		(60)
Control	10/10			
Pmx*	0/10	5/10	8/10	
		Polymyxin		30
Control			0/10	
Pmx*			"	
		Saline		-
Control				
Pmx*	35/35			

* Challenge consists of respective drug dose as a mixture containing a sublethal dose (3.5 ug/g) of polymyxin B.

A comparison of ACTH with STH and TSH, respectively, indicated that ACTH was the only one of these protein hormones from the adenohypophysis which had

a demonstrable effect on survival ratios or was an effective dye releaser. Chymotrypsin, another protein with biological activity, had no significant effect on survival or dye release. However, clupein, a basic protein derived from a nucleoprotamine, proved to have marked effect on survival ratios and also proved to be an effective releaser substance. The non-specificity of the ACTH effect on polymyxin tolerance can thus be seen and it would appear that this effect is related to the basic protein character of ACTH. Moreover, neomycin, a basic polypeptide-like antibiotic substance, also had a definite effect on survival ratios at dose levels which were not lethal to control mice. This substance also had a pronounced dye releaser activity.

Toluidine blue, a basic thiazine dye with a well known affinity for heparin either in vitro or in vivo, also potentiated the toxicity of polymyxin. This observation gives further support to the idea that the potentiation of polymyxin toxicity by these basic substances is a result of their competition with polymyxin for certain non-specific bindings with acidic polyelectrolytes of the tissues. Furthermore, it is obvious from the table that polymyxin itself is the most active potentiator of polymyxin toxicity and that, like all other substances found to potentiate polymyxin toxicity, it also has an effective dye release activity.

This concept of ACTH-mucopolysaccharide interaction in the tissues is further supported by the results shown in table 5.

Table 5

Effects of ACTH and Other Basic Drugs on Tolerance of Mice to Polymyxin B

Challenged Groups	Drugs Added to Challenge Dose (25 ug/gm)	Number of Survivors
Lethal polymyxin dose plus Heparin*		
1. Polymyxin B	Control	0/15
2. Pmx-Heparin	Control	15/15
3. Pmx-Heparin	ACTH	1/10
4. Pmx-Heparin	Clupein	0/10
5. Pmx-Heparin	Toluidine blue	0/10

* Intravenous injection of 3.0 ug polymyxin B/gm alone (1) or as a mixture with the test substances (2-5).

In this study a lethal dose of polymyxin was employed (5.0 ug/gm) and this could be neutralized by 1.5 ug/gm heparin. In this situation as before, ACTH potentiated the toxicity of polymyxin, thus neutralizing the protective effect of the heparin. Toluidine blue, as well as clupein, also reversed the protective effect of heparin on the tolerance of mice to polymyxin intoxication. Thus the physiologic state of the tissue, with respect to polymyxin B can be related to the respective activities of two naturally occurring substances of the body: ACTH and heparin. Since polymyxin is a stress-producing substance, ACTH further enhances the stress situation, whereas heparin has an anti-stress effect. Heparin serves to supplement the naturally occurring mucopolysaccharides of the tissues whose functional activity can be overwhelmed by various substances endogenously released (ACTH, etc.) or administered (neomycin) during stressful conditions.

V. Factors influencing the toxicity of a snake venom, etc. It has been found that either clupein or toluidine blue will enhance the toxicity of compound 48/80. Thus these well-recognized anti-heparin substances can potentiate the lethal effects of two diverse histamine releaser agents: compound 48/80 and polymyxin B.

Toluidine blue also potentiated the lethal effects of the venom of Russell's viper in mice. Further studies of this effect showed that heparin as well as other acidic mucopolysaccharides, could reduce the toxicity of this snake venom. The studies were carried out in a manner similar to those above with polymyxin and ACTH. These observations further support the theory that acidic mucopolysaccharides have an important role in the resistance to stress. It has been shown that alterations in the concentration as well as in the physical and chemical states of these substances produce alterations in resistance to various stress-producing substances.

Chlorpromazine, a basic substance with tranquilizing properties, forms complexes with heparin in vitro. This drug, when injected subcutaneously, causes a marked degranulation of local mast cells. However, it does not markedly inter-

fere with the anticoagulant effect of heparin nor does heparin interfere with its tranquilizing effects. This drug has a phenthiazine structure and its effects were compared to those of the thiazine dye, toluidine blue, on the toxicities of compound 48/80, polymyxin B and Russell's Viper venom. The results are shown in table 6.

Table 6

Effect of Toluidine Blue and Chlorpromazine on Tolerance to Sublethal Doses of Various Toxic Drugs.*

Drugs and Doses (γ/gm)	Survivors			
	Control	Compound 48/80 2.0 γ/gm	Polymyxin B 3.5 γ/gm	Russell's Viper Venom 0.3 γ/gm
Toluidine Blue				
Control	-	10/10	10/10	10/10
2.5	-	8/10	-	-
5.0	-	6/10	10/10	-
10.0	-	4/10	5/10	8/10
15.0		0/10	0/10	4/10
20.0	10/10	-	-	0/10
Chlorpromazine				
Control	-	10/10	10/10	10/10
2.5	-	10/10	10/10	-
5.0	-	7/10	8/10	10/10
10.0	-	3/10	1/10	5/10
15.0	10/10	-	-	0/10

* All materials injected as mixtures of appropriate drugs contained in a volume of 0.25 ml saline administered by i. v. route. Deaths occurred within 1 to 15 min.

It is shown that the effects of these basic substances parallel each other in enhancing the toxicity of the various challenge agents. Heparin can reduce the toxic effects of both of these agents by complexing with the toxic challenge agents

by complexing with the toxic challenge agents and with the toluidine blue. It is evident, therefore, that various therapeutic drugs may potentiate the toxicity of various other substances. Toxicity can therefore be a composite of responses to diverse substances and "heparin" may alleviate this condition either directly (ACTH, Neomycin plus polymyxin) or indirectly (chlorpromazine plus polymyxin, etc.). This study is being continued with regard to the nature of biological substances with which chlorpromazine may interact or compete. Studies in other laboratories have indicated that it may interfere with the activity of 5-OH-tryptamine.

Basic Amines. The theory that toxicity is a composite of many single effects was investigated with regard to histamine. This substance is usually credited with being the mediator of inflammation, anaphylaxis, etc.. However, mice are notoriously resistant to the pharmacological effects of this substance. Various naturally occurring tissue amines may release histamine. This suggests that they may act as "replacers" of histamine in its fixed state in the tissues. Thus the active concentration of histamine may be much greater than suggested by its total concentration in the tissues. It was found that spermidine, agmatine, putrescine and cadaverine can significantly enhance histamine toxicity (table 7).

Table 7

Effect of Natural Tissue Amines on Histamine Toxicity

Dose of Amines Used in Challenge	Histamine Dosage*			
	Control	55 γ/gm	60 γ/gm	70 γ/gm
Putrescine 100 γ/gm	+	+	+	+
Cadaverine 120 γ/gm	+	+	+	+
Spermidine 40 γ/gm	+	+	+	+
Agmatine 80 γ/gm	+	+	+	+
Survivors	9/10	7/10	3/10	0/10

* Lethal dose of histamine 275 γ/gm.

It is shown that the amounts of the individual amines used in this study were non-toxic and that the mixtures of these amines (without histamine) were relatively non-lethal (80% survival). The amount of histamine required to produce lethal intoxication (0% survival) in mixture with the other amines was [redacted] one-fifth of the histamine LD_{50} dose by the intravenous route. The results suggest that histamine toxicity in mice can be a natural phenomenon under conditions in which other tissue amines are also released. However, it would be an oversimplification to assume that the histamine was the sole mediator of these toxic states. Since various other substances are also released as the result of tissue injury and may contribute to the toxic effects of histamine, therapy directed towards any one constituent could alleviate the stress condition. An example of this is the use of heparin in intoxications of mice resulting from overdosage with compound 48/80 or polymyxin B. Heparin readily reverses the intoxications produced by these histamine releaser substances, yet it does not markedly alter intoxications resulting from administrations of histamine itself.

VI. Fate of administered heparin and x-irradiation studies. A large number of studies have been carried out in this laboratory showing that fibroblasts of the loose connective tissue ingest and temporarily store this metachromatic substance. A study was carried out to correlate this effect with the loss of heparin from the blood as measured by the blood coagulation time.

Administered heparin (1.25 mg) prolongs blood coagulation time of mice over a period of 6 to 7 hours. During this time it is accumulated in the interstitial tissue by the fibroblasts and stored in their cytoplasms as metachromatic granules. This material is digested during the subsequent 36 to 48 hours. These results are of interest since the current ideas on the fate of heparin consider that it is broken down by "heparinase" in the liver and/or excreted as uroheparin by the kidneys.

A number of studies in other laboratories have considered that prolonged co-

agulation times of blood following irradiation are due to release of heparin from the mast cells to the blood. In our study, x-irradiation (350 r, 650 r) failed to alter coagulation times of control or heparin-treated mice. In the latter group, the animals were irradiated 7 to 8 hours after heparin treatment, at which time the heparin was not detectable in the blood and was concentrated in the loose connective tissue. Since fibroblasts readily ingest "free" heparin or bound heparin (mast cell granules) it would be expected that heparin released from an injured cell would appear in the blood only with difficulty.

However, the x-irradiation did appear to reduce the ability of some cells to metabolize their ingested heparin. Preliminary observations also suggested that pretreatment with heparin could prolong survival time of mice given a lethal dose of x-irradiation (650 r). These results are being investigated further with the idea that heparin in the tissues serves to bind with various noxious substances which may be released as the result of x-irradiation injury. Administered heparin could thus increase the tissue capacity to deal with these substances if it is allowed to accumulate in the tissues prior to injury and thereby not be acting as an anticoagulant at the time of injury.

VII. Steroid sulfates. A preparation of sulfated estrogenic hormones known as "Prem" has been tested for its effects on the lethal toxicity of various substances and it has been found that it will protect mice against a lethal dose of the histamine releaser, compound 48/80, but not polymyxin B. Additional studies with the purified steroidal constituents of this preparation have been carried out, but none of the sulfated steroids used had any marked effect on compound 48/80 intoxication.

The effects of "Prem" on anaphylaxis, histamine intoxication, etc., have been studied with no marked effects observed. This mixture will form precipitates in vitro with various cationic substances ranging from ACTH to histamine. However, more often than not it tends to potentiate toxic effects rather than

inhibit them. This may be due to the formation of relatively insoluble precipitates within the blood stream. Only compound 48/80 toxicity is significantly inhibited.

VIII. Sulfated macromolecules. Two fractions of polyethanol sulfates have been briefly tested. One fraction (10-15,000 mol. wt.) which reportedly activates plasma lipase, protects against compound 48/80 toxicity and is ingested and stored by fibroblasts. The other fraction (5-10,000 Mol. wt.) reportedly has little effect on plasma lipase, does not protect against compound 48/80 and does not readily appear as granules in fibroblasts.

IX. Nucleic Acids. Desoxynucleic acid (DNA) and pentose nucleic acid (PNA) are readily ingested by fibroblasts when these macromolecules are complexed with compound 48/80, but not as "free" substances. In related studies it was found that polymyxin B (a basic polypeptide) as well as salmine and clupein (protamines) also serve to facilitate this sequestration of nucleic acids by fibroblasts. DNA is ingested more rapidly under these conditions than is PNA. It was found that approximately 156 cells/mm^2 of loose connective tissue showed uptake of 48/80-DNA within 1 hour vs. none in tissue ingected with DNA alone. Similarly, 119 cells/mm^2 showed 48/80-PNA uptake within 2 hours vs. none for PNA alone.

Further studies showed that pretreatment of mice with single graded doses of either DNA or PNA could provide protection vs. a lethal dose of compound 48/80 given by this same route 30 minutes later. Calculations of results showed that DNA was approximately 5 times more active in conferring protection than was RNA. The 50% protective dose of DNA was approximately 10 ug/gm whereas that of PNA was 50 ug/gm. The results suggest that DNA and PNA behave somewhat differently in the tissues and not simply as phosphorylated macromolecules.

In summary, nucleic acids can interact with compound 48/80 in vivo and this complex can be sequestered by fibroblasts and deposited as granules within their cytoplasms. The importance of the nucleic acids in various aspects of

biology and the marked histamine releaser activity of compound 48/80 imply the fundamental nature of this phase of micellophagosis. The fibroblastic uptake of a nucleic acid and the passage of nucleic acid materials thru the cell membrane in virus infection and transduction experiments may be very similar phenomena. The role of a substance such as compound 48/80 in enhancing passage of nucleic acids into cells merits further consideration, since it could be highly significant with respect to carcinogenesis.

Discussion

The roles of histamine and heparin in the tissues are fundamental in the maintenance of the physiologic state. Heparin binds relatively large amounts of tissue histamine (mast cells) and in this state can serve as a readily available substance to bind with other agents for which it has a stronger affinity (e.g., histamine releasers). The toxicity of histamine can vary according to the amount of histamine released as well as to the conditions of release. It has been shown in this study that histamine is markedly more toxic in the presence of other tissue amines than when alone. Likewise, the toxicity of a histamine releaser is related to the amount of heparin available to bind with it. Therefore, the response of an organism or its tissues to a given amount of a stress stimuli is related to numerous factors and the response can be lacking (tolerance) or exaggerated (hypersensitivity). Specific therapy directed to any one component of the stress-producing complex may be sufficient to alleviate the stress situation. Thus many agents, antihistaminics, adrenocortical hormones, heparin, etc., may serve as anti-stress substances - each acting in its own specific capacity. It is therefore considered important to explore the nature of their respective activities. In this way, the therapeutic values of these drugs can be exploited more specifically and therefore, more effectively.

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