Potentiation of Polymyxin B Toxicity by ACTH.*† (23509)

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The degree of polymyxin B toxicity has been found to be a function of the concentration of heparin in the tissues and of the dose of polymyxin employed(1). The basic polypeptide, polymyxin B, and the acidic mucopolysaccharide, heparin, form a complex *in vitro* which does not readily dissociate *in vivo*. This complex can be sequestered by fibroblasts and deposited as intracytoplasmic metachromatic granules (quasi-mast cells)(2). Acute polymyxin toxicity thus appears to be due to the amount of polymyxin in excess of that amount fixed by endogenous acidic mucopolysaccharides of the tissues. It has been postulated that heparin can act in the tissues as an ion exchanger(3). The enhanced polymyxin B tolerance of heparin-treated mice led to the assumption that the natural capacity for polymyxin tolerance is dependent on an ion exchange function of the tissues. The ability of an animal to tolerate polymyxin is represented as a function of the capacity of acidic macromolecules in its tissues to exchange naturally bound substances for polymyxin. The release of histamine *in vivo* by polymyxin(4) and the degranulating effect of polymyxin on the heparin- and histamine-containing mast cells of connective tissue(5) would support this assumption. As is well known, the capacity of an ion exchanger is a quantitative measure of its ability to exchange one substance for another and, during the course of its operation, this capacity becomes progressively smaller. In the following study the possibility has been examined that a naturally occurring substance which can form a complex with heparin may decrease the capacity of the tissues to fix polymyxin and thereby relatively decrease the capacity of polymyxin tolerance in the animal. The results obtained with the basic protein, adrenocorticotrophic hormone (ACTH) are in accord with this idea and indicate that ACTH can non-specifically potentiate polymyxin toxicity in a dose-response manner.

Materials and methods. Complex formation. The formation of heparin complexes with various drugs was determined as follows: The system consists of 120 μg of the dye, toluidine blue (G. Grubler & Co.), in complex with 50 μg of heparin (117 u/mg, The Upjohn Co.)‡ in a series of tubes. Graded amounts of the test substances were added to make a final volume of 6 cc, all substances having been dissolved in deionized water. Toluidine blue and heparin rapidly form a complex which has an optimal absorption at 540 mμ in contrast to the optimal absorption of "free" toluidine blue at 630 mμ(6). The dissociation of the toluidine blue-heparin complex is enhanced in the presence of any of a number of basic substances which have an affinity for heparin. The dissociated dye is freely dialyzable, and, at optimal proportions, the newly formed drug-heparin complex forms a definite precipitate. The amount of free dye was determined with a Coleman Jr. Spectrophotometer at a wave length of 630 mμ and the results plotted against the respective amounts of basic substance used. The relative affinities of various substances for heparin were then compared.

Toxicity studies. All materials for injection were prepared in isotonic, pyrogen-free, sterile saline and were injected by the intravenous route in a volume of 0.25 ml. Polymyxin B (Aerosporin)‡ dose was based on the biological equivalent of the 50 mg polymyxin B standard (500,000 units of activity). CBA mice, 2 to 3 months of age and ranging in weight from 20 to 22 g, were challenged with a standard *sublethal* dose of polymyxin (3.5 μg/g body weight) or mixtures of this stand-

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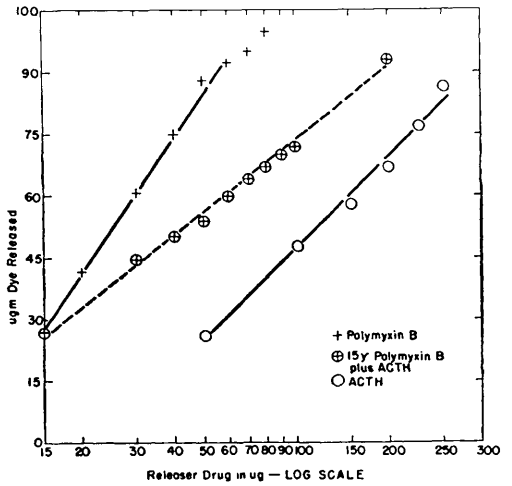

FIG. 1. Comparison of dye release activities of 1) graded amounts of polymyxin B, 2) a constant amount of polymyxin B (15 μg) with graded amounts of ACTH and 3) graded amounts of ACTH. Note additive effect of ACTH on release of toluidine blue from heparin complex by polymyxin B.

ard polymyxin dose with a given amount of one of the test substances. Control mice were given the individual drugs in the same amounts and manner. Toxicity was determined on the basis of number of animals surviving for 24 hours following challenge. However, under the conditions of this study deaths usually occurred within 5 minutes after injection. The substances tested for their heparin affinity as well as for their effect on polymyxin toxicity were: ACTH (ACTHAR, H.P., lyophilized), STH (Somatotropin, lyophilized), TSH (Thyrotropar, lyophilized) and Chymotrypsin from Armour and Co.,‡ clupein sulfate (K and K Laboratories) and neomycin sulfate (665 μg/mg, Nutritional Biochemicals Corp.) and toluidine blue.

Results. In vitro. The toluidine blue-heparin complex was used as an *in vitro* model system to simulate a readily dissociable state of acidic macromolecules in the tissue. The interaction of polymyxin with this heparin complex as evidenced by the release of toluidine blue is shown in Fig. 1. Since the dissociated dye is freely dialyzable and, at optimal proportions, the polymyxin-heparin complex is visible as a definite precipitate, the polymyxin can be designated in this system either as a "releaser" of toluidine blue or as a "binder" of heparin. The concomitant formation of a heparin complex with the polymyxin releaser is also indicated by the markedly reduced toxicity of polymyxin when complexed with heparin either *in vitro*(7) or *in vivo*(2). Therefore, the release of dye is considered as indirect evidence of the formation of a complex between the "releaser" and heparin.

As indicated in Fig. 1, the amount of dye released is proportional to the log concentration of polymyxin within the measured range of 15 to 80 μg polymyxin per 6 cc of the test system. It is also shown in Fig. 1 that with a constant amount (15 μg) of polymyxin and graded amounts (15-100 μg) of ACTH, the total amount of dye released/μg of polymyxin is progressively greater but proportional only to the ACTH concentration. Thus the release of dye by polymyxin is of a non-specific nature. Actually, as shown in Fig. 1, ACTH also has a dye releasing activity which is consistent with an ability to bind with heparin (8). Therefore ACTH has an *additive* effect on this *in vitro* dye releasing activity of polymyxin. Since the biological effects of polymyxin and ACTH are apparently so dissimilar, this additive effect of ACTH would be manifested *in vivo* as a *potentiation* of a polymyxin effect in the tissues (*i.e.*, toxicity). In other words, ACTH could compete with polymyxin for binding with acidic mucopolysaccharides in the tissues and in this manner serve to deplete the total number of active sites otherwise available for the fixation of polymyxin. The net effect of this competition would be to increase the amount of polymyxin free to act at other sites in the tissues and would be equivalent to the effects of larger doses of polymyxin.

In vivo. To evaluate the possibility that ACTH could competitively interact with polymyxin "binding sites" in the tissue, mixtures containing a standard *sublethal* dose (3.5 μg/g) of polymyxin and graded amounts (0-37.5 μg/g) of ACTH were used as intravenous challenges for a number of groups of mice. The experimental groups consisted of 10 animals each. The intravenous route was used for challenge since this is the most sensitive method for eliciting the toxic effects of polymyxin.

In accord with the assumption made, in-

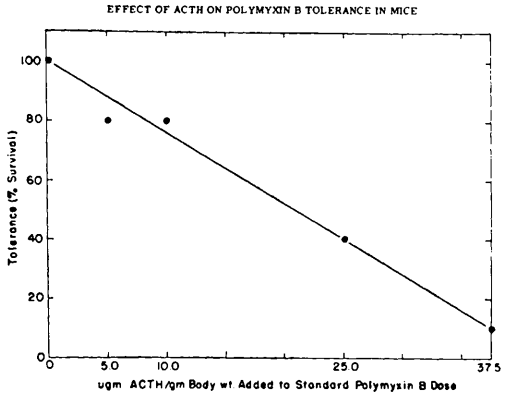

FIG. 2. Effect of ACTH on polymyxin B tolerance of 11 weeks old CBA mice (10 mice/group). Animals were injected intravenously with a standard *sublethal* dose of polymyxin B containing graded amounts of ACTH (abscissa). Note that tolerance decreases as ACTH dose increases.

creasing doses of ACTH should progressively decrease heparin-like sites in the tissues otherwise available for interaction with a given amount of polymyxin. Concomitantly, there should be an increase in the relative amount of polymyxin available to interact with more vital sites or activities in the tissues. The postulated ACTH effect should therefore be measurable in terms of a decreased polymyxin tolerance relative to the ACTH dose. In Fig. 2, the per cent survival has been plotted against the respective dose of ACTH (μg/g) contained in the polymyxin-ACTH mixture. It can be noted that mice receiving the control polymyxin dose had a 100% survival. In related experiments a total of more than 35 mice have received this standard polymyxin dose without lethal effects (see Table I). However, those animals given 5 to 10 μg ACTH/g with the polymyxin dose had only an 80% survival; 25 μg ACTH/g: 40% survival; and 37.5 μg ACTH/g: 10% survival. Larger amounts of ACTH (50 μg/g) have been found to decrease the survival to 0%. Control doses of ACTH alone had no obvious toxic effects (see Table I). These results indicate that although the interaction of ACTH with tissue constituents does not of itself induce lethal toxicity, this interaction can reduce tolerance for polymyxin by interfering with tissue mechanisms necessary for dealing with this latter drug. The net effect is a potentiation of polymyxin toxicity by ACTH.

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

Group	Treatment and survival ratios — Drug doses (μg/g) 25	12.5	6.25	50% dye release activity (μg)
		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 μg/g) of polymyxin B.

Specificity. 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 μg/g) of the test substances 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 μg) 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 I.

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* (6) or *in vivo*(9) 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.

Discussion. A potential affinity of ACTH for various acidic constituents of the tissues has been discussed(10) with emphasis on the significant relationship between the basic character of ACTH and the acidic nature of the basophilic cells of the adenohypophysis. Reports that ACTH complexes with heparin *in vitro*(8) and possibly *in vivo*(11) give further support to this idea. Similarly, the basic character of polymyxin B, and its affinity for phosphorylated constituents of the bacterial cell(12) as well as its formation of complexes with heparin either *in vitro* or *in vivo*(2) indicate that it also has a potential for interacting with various acidic constituents of the tissues. Obviously, such interactions would be partially non-specific and this would imply that ACTH and polymyxin B could non-specifically compete for acidic binding sites in the tissues. This assumption is supported by the observations in the present study that both ACTH and polymyxin enhance the dissociation of a toluidine blue-heparin complex (*i.e.*, heparin binding) *in vitro*, that they have an additive effect on this dissociation, and that ACTH potentiates polymyxin toxicity *in vivo*. These observations as well as those by other workers indicate that ACTH can participate in biological activities of an extra-adrenocortical nature. For instance, ACTH has been found to inhibit the conjugaton of cortisol by the liver(13). With regard to the interaction of ACTH with other basic substances, recent studies by other laboratories have indicated that the basic polypeptide, pitressin, has a measurable and perhaps specific ACTH releaser activity *in vivo*(14), that the basic polypeptide, glucagon, can exert a sparing effect on demonstrable ACTH in adenohypophyseal minces(15) and conversely, that alpha corticotrophin, etc., can potentiate the activity of otherwise ineffective amounts of glucagon *in vitro*(16). The effect of ACTH on polymyxin toxicity reported here would appear to be only one of a number of examples in which a biological activity of a given basic substance is markedly enhanced by the presence of another. In the present instance, the effects would not require the direct intervention of specific enzymatic processes for their demonstration and could be most easily explained as the result of a competition between the basic substances for the same acidic sites in the tissues. Thus, with a limited number of acidic sites capable of binding polymyxin, the amount of polymyxin bound would be relative to the concentration of other substances capable of competing with polymyxin for these sites. Since polymyxin toxicity is dose dependent and the depletion of non-specific binding sites in the

tissues would increase the relative amount of polymyxin available for interaction with more vital sites or activities in the tissues. ACTH enhances the toxicity of polymyxin B. In this manner, an otherwise sublethal amount of polymyxin becomes a fatal dose.

The reduction of polymyxin tolerance by clupein, neomycin or toluidine blue (which are structurally unrelated to ACTH, polymyxin or to each other) indicates the non-specificity involved in the effects of such substances on the *capacity* of the tissues to tolerate polymyxin. The effects of these substances on polymyxin tolerance can be correlated by their common affinity for heparin *in vitro*. Heparin as well as other acidic substances such as chondroitin sulfate B or any of a variety of chemically sulfonated macromolecules have been found to enhance the tolerance of these animals for polymyxin(1.7). Theoretically, polymyxin tolerance in the tissues can be described as a function of endogenous heparin. The term "heparin" is used here in a generic sense to include those tissue substances capable of complexing with polymyxin such as other sulfonated polysaccharides. It has been proposed that the mechanism in the tissues whereby heparin interacts with polymyxin, etc., is related to the capacity of heparin to serve as an ion exchanger (*i.e.*, a readily dissociable state of heparin)(3). In this respect, heparin can become relatively unavailable for interaction with polymyxin if the capacity of this exchanger has been reduced as a consequence of its interaction with other substances which also have a strong affinity for this tissue mucopolysaccharide. The linear dose effect of ACTH on polymyxin tolerance can readily be explained on this basis. Therefore, the capacity to tolerate a given amount of polymyxin can be measurably altered in one direction or another (tolerance versus toxicity) by certain naturally occurring substances in the tissues (heparin versus ACTH).

It may be held unlikely that a sufficient amount of endogenous ACTH is present following stress to produce toxicity according to the point of view given here. Basic substances acting as stressors and the accumulated basic polypeptides released from injured cells could all compete for sulfonated macromolecules and this summation of complexes could produce stress by exceeding the ion exchange capacity of the tissue. This possibility has been explored and will be reported later.

Summary. An *in vitro* technic has been used to detect the interaction of various basic substances (ACTH, polymyxin, clupein and neomycin) with heparin, an acidic mucopolysaccharide of the tissues. It was observed that substances effectively interacting with heparin in this *in vitro* system markedly potentiated the toxicity of polymyxin B *in vivo*. It has been suggested that polymyxin tolerance is dependent on an ion exchange function of heparin-like constituents of the tissues and that the capacity for tolerance can be reduced by substances which have a strong affinity for these heparin-like constituents.

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