THE CARDIOTOXIC ACTION OF PREPARATIONS CONTAINING THE OXYGEN-LABILE HEMOLYSIN OF STREPTOCOCCUS PYOGENES

I. Increased Sensitivity of the Isolated Frog's Heart to Repeated Application of the Toxin

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Among the substances known to produce systolic contracture of the frog's heart are: (*a*) vegetable poisons, notably the glycosides of the digitalis group (1) and saponins (2); (*b*) animal venoms such as those of the toad (3), the cobra, and other snakes (4); (*c*) certain bacterial toxins (5); (*d*) oxidizing agents (6). Some of these substances are also hemolytic, the association of hemolytic activity with cardiotoxic substances appearing to be rather frequent and widespread in naturally occurring materials.

The toxic effects of the most thoroughly studied bacterial toxins, such as tetanus toxin (tetanospasmin) and diphtheria toxin, develop only after a latent period of considerable length, while the toxic effects which follow the intravenous injection of the toxins of *Clostridium welchii*, *Cl. septicum*, and staphylococcus develop almost immediately. It may be noted that all of the toxins of the latter group are hemolytic *in vitro* while the slow acting toxins are not hemolytic.

Dale, in an appendix to a paper by Robertson (7), suggested that the toxin of *Cl. septicum* has two effects on the cardiovascular system of rabbits: a pressor action and also a direct toxic action on the heart muscle. Lautenschläger (5) in a more extended study confirmed Dale's suggestion of a cardiotoxic action. Using unconcentrated filtrates of *Cl. septicum* cultures he analyzed the circulatory collapse that follows intravenous administration of the toxin and found that death is due to the cardiac action of the toxin. He was impressed by the superficial resemblance of the action of the toxin to that of the digitalis glycosides on the mammalian heart and the isolated frog's heart. The pharmacology of the toxins of the gas gangrene organisms was further studied by Buttle and Trevan (8) and by Kellaway, Reid, and Trethewie (9). The latter authors verified the correctness of Dale's suggestions and attributed the complex circulatory effects of the toxin partly to a specific cardiac action and partly to constriction of pulmonary and coronary vessels. In addition to the observations on *Cl. septicum* toxin, staphylococcal toxin has been found by Kellaway, Burnet, and Williams (10) to produce acute circulatory symptoms and by Dingle, Hoff, Nahum, and Carey (11) to produce acute myocardial damage. The circu-

latory symptoms according to Feldberg and Keogh (12) can be attributed to the liberation of histamine.

While engaged in a study of the pharmacological actions of the toxins of the anaerobic spore-forming bacilli, we confirmed the observations just cited of Lautenschläger on the frog's heart. It appeared worth while to investigate the possible association of hemolytic and cardiotoxic activity of the hemolysins of bacteria other than the sporulating anaerobes. The oxygen-labile hemolysin of *Streptococcus pyogenes* was selected because it has been studied more thoroughly than most other bacterial hemolysins. The observations of the action on the isolated frog's heart of this hemolysin (or of a substance closely related to it) form the subject of the present communication.

Methods and Materials

Most of the experiments were performed in the months of July, August, and September on isolated hearts of *Rana pipiens* prepared according to Straub's technique (13). A straight Fühner cannula with a capacity of 1.2 cc. was used.

The physiological solution used in all experiments had the following composition: NaCl 0.65 per cent, KCl 0.01 per cent, $CaCl_2$ (anhydrous) 0.01 per cent. The pH of the solution was maintained at 7.4 with phosphate buffer in a final concentration of 0.00345 M. A fine stream of oxygen was bubbled through the fluid in the cannula. All experiments were conducted at room temperature.

Preparation of Hemolysin-Containing Fraction of Culture Supernate Used in the Experiments.—The smooth variant of *Streptococcus pyogenes*, strain C203S, was grown in 10 liter cultures according to the method described by Bernheimer, Gillman, Hottle, and Pappenheimer (14). This method was employed because the biologically active substances produced by the streptococci can be obtained in good yield and because the isolation of these substances is facilitated by the use of a medium which is free from extraneous substances of high molecular weight.

After frequent neutralization of the acid formed over a 20 hour period of growth, the cultures were refrigerated overnight and centrifuged on the following day. The supernatant fluids, to which were added a few drops of toluene, were concentrated five- to eightfold in large cellophane sacs suspended in a current of air. The concentrates were dialyzed overnight against half-saturated ammonium sulfate in the cold, followed by dialysis against running tap-water. The concentrates were next dialyzed against saturated ammonium sulfate in the cold for 4 to 6 days at the end of which time precipitation was complete. The precipitates were collected by centrifugation and were stored in the refrigerator under a small volume of saturated ammonium sulfate. The over-all reduction in volume was approximately 300-fold.

The hemolytic potency of the concentrates was tested in a manner similar to that described elsewhere (15) except that the hemolysin was activated with 1 per cent cysteine before dilutions were made. The hemolytic unit is the amount of hemolysin which liberates half the total hemoglobin contained in 1 cc. of a 0.7 per cent suspension of washed human erythrocytes, in a final volume of 2 cc. The average hemolytic potency of the concentrates was 100,000 units per cc. It was observed that the erythrocytes developed increased resistance to lysis by streptococcal hemolysin when the cell suspension was stored for more than a day or so in the cold. For this reason, freshly prepared cell suspensions were used whenever possible.

Preparatory to testing on the frog's heart, a portion of the concentrated culture supernate was dialyzed free of ammonium sulfate, centrifuged at high speed in order to remove residual cells, and stored in the cold without further purification. The dialyzed concentrates undoubtedly contained a mixture of streptococcal products in addition to hemolysin and for this reason they are noncommittally referred to as SPA (streptococcal preparation A). As will be shown in a later section the substance in SPA which acts on the frog's heart is activated by cysteine. Unless otherwise indicated, SPA in a concentration of 1:100 was always activated in the cold with cysteine (1:1000) for 10 minutes. The activated SPA was further diluted with Ringer's solution so that the concentration of the cysteine in the solution finally applied to the heart was 1:2500.

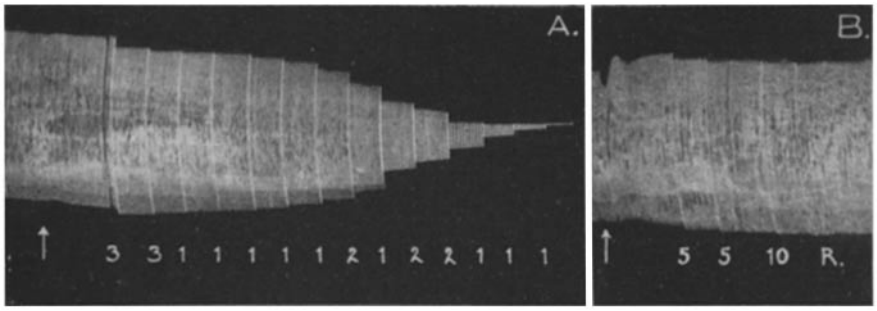

FIG. 1. Comparison of the effects of *Clostridium septicum* toxin and streptococcal preparation A (SPA). Isolated frog's heart (Straub) suspended on Fühner's cannula: Ringer's solution 1.2 cc. Kymographic record of amplitude of ventricular contraction.

(A) The effect of *Clostridium septicum* toxin: At arrow *Clostridium septicum* toxin 1:35 added. Figures indicate in minutes the periods of arrest of the drum.

(B) The effect of streptococcal preparation A: At arrow SPA 1:25 in cysteine 1:1000 added. R, washing with Ringer's solution. Figures indicate in minutes the periods of arrest of the drum.

EXPERIMENTAL

Comparison of Effects Produced by Cl. septicum Toxin and by SPA.—

The supernatant fluid from a culture of *Cl. septicum* grown in a peptone-free medium (16) was applied to the heart in a dilution of 1:35. After 8 minutes, the systole and diastole started to decrease (Fig. 1A), and continued progressively to do so during the next 20 minutes, at the end of which time the ventricle had stopped beating midway between systole and diastole. The auricles were distended with fluid and continued to beat after the ventricle had stopped.

These observations are similar to those recorded by Lautenschläger (5).

The dialyzed streptococcal concentrate prepared as already described was diluted 25 times in Ringer's fluid containing cysteine in a concentration of 1:1000. After 2 minutes at room temperature this solution was administered to the heart and allowed to remain in the heart for 23 minutes. It was found to have no effect other than a slight increase in amplitude of contraction (Fig. 1B). This observation has been repeated in the course of several hundred

experiments in which concentrations of SPA varying from 1:25 to 1:1000 were used. A small proportion of the hearts, less than one in twenty, unpredictably developed systolic contracture, indistinguishable from that described in the next paragraph.

Effect of Repeated Application of SPA—Although a single dose of SPA was usually without effect on the frog's heart, an entirely different result was obtained when a second dose of SPA was brought into contact with a heart which had been treated previously with SPA. The response of the heart to *two* doses is illustrated by the following experiment.

The introduction into the heart of SPA in a concentration of 1:25 was without effect. At the end of 5 minutes the solution was removed, and during the next 10 minutes the heart was twice washed with Ringer's solution. Next, a second dose of SPA identical with the first was introduced into the Führer cannula. Within 30 seconds decreased relaxation of the ventricle occurred (Fig. 2A) and within 90 seconds the heart underwent complete systolic contracture.

The experiment shows that exposure of the heart to a single dose of SPA modified the heart in such a way that it went into systolic contracture when SPA was applied a second time. There now arose the question of whether this effect could be explained by a simple summation of two subthreshold doses or whether the first dose "sensitized"[1] the heart to the second.

A heart was exposed to activated SPA in a concentration of 1:500 (Fig. 2B). After 5 minutes, the heart was washed with two portions of Ringer's solution, and a second dose of activated SPA, 1:500, was applied. The heart rapidly developed systolic contracture.

In this experiment the total quantity of SPA applied to the heart was 0.004 cc. In the experiment represented by the tracing reproduced in Fig. 2A a single application of 0.04 cc. failed to produce systolic contracture. Inasmuch as two applications of SPA accomplished an effect which was not produced by a single application representing ten times the sum of the two doses, it is clear that the phenomenon is a true sensitization rather than the result of cumulative action. Further indication that the phenomenon described consists in a true sensitization is afforded by the observation that even after repeated washing during a period of 3 hours, the heart underwent contracture when exposed to a second small dose of SPA.

As can be seen in Fig. 2B, the contracture produced by the second administration of activated SPA was followed by spontaneous recovery of the heart. This spontaneous recovery which is due to the rapid inactivation of the hemolysin is dependent upon the dose of the activated toxin used and is best observed when a small dose of toxin is applied to the heart (compare Figs.

[1] The word "sensitize" is not intended to connote sensitization in the immunological sense, but is here employed in accordance with general biological usage, that is, to render susceptible or responsive.

2A and 2B). When a third dose of activated SPA was administered to the recovered heart, systolic contracture again developed (not shown in figure).

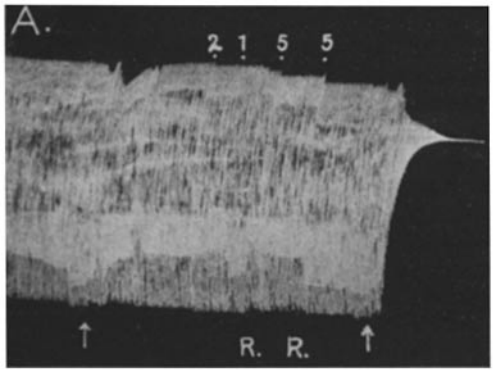

FIG. 2A

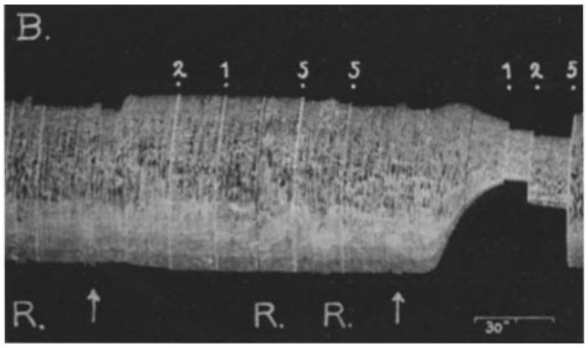

FIG. 2B

FIG. 2. Sensitization of isolated heart to streptococcal preparation A (SPA). Isolated frog's heart (Straub) suspended on Fühner's cannula: Ringer's solution 1.2 cc. Kymographic record of amplitude of ventricular contraction.

(A) At arrows—SPA 1:25 in cysteine 1:2500. R, washing with Ringer's solution. Figures indicate in minutes the periods of arrest of the drum.

(B) At arrows—SPA 1:500 in cysteine 1:2500. R, washing with Ringer's solution. Figures indicate in minutes the periods of arrest of the drum.

Properties of the Active Principle of SPA.—Two characteristics of the substance which acts on the heart are indicated in the method of preparation of SPA, namely that it is non-dialyzable and that it is salted out by saturated ammonium sulfate. Evidence showing that the cardiotoxin is actually a product of the streptococcus is presented in a subsequent section.

The active principle was further characterized by the results of a series of experiments the description of which follows.

The concentrations of SPA and of cysteine used were the same in the first (sensitizing) dose and in the second (contracture-producing) dose. The two doses were applied 15 minutes apart, and each was allowed to remain in the heart for 5 minutes. The heart was washed twice with Ringer's solution between the first and the second dose.

1. Heat Lability.—

Several cubic centimeters of the streptococcal preparation were diluted tenfold in Ringer's solution and heated for 10 minutes at 60° C. The solution was cooled and diluted tenfold with Ringer's solution containing cysteine (1:1000). After 10 minutes the solution was further diluted with 1.5 volumes of Ringer's fluid making the final concentration of SPA 1:250. Two doses of this dilution were found to be without effect on the heart while the second of two administrations of an unheated control solution containing streptococcal preparation in a concentration of 1:300 produced systolic contracture.

This experiment, summarized in Table I, shows that the substance which acts on the heart is relatively thermolabile, all or most of its activity having been destroyed by exposure to 60° C. for 10 minutes.

2. Activation by Cysteine.—The purpose of this experiment was to find out whether or not the substance acting on the heart was activated by cysteine.

A fine stream of oxygen was bubbled for 8 minutes through several cubic centimeters of the streptococcal preparation diluted 1:10. Two doses of the oxygen-treated material failed in a final concentration of 1:100 to affect the heart. In order to see whether the oxygen-treated preparation could be activated by cysteine, it was incubated in a concentration of 1:20 with cysteine 1:250 for 5 minutes in the cold. Complete systolic standstill was produced by this solution diluted to a final concentration of 1:200. This concentration, it may be noted, is only one-half that of the solution tested prior to activation with cysteine, the latter having been found to be inactive.

This and similar experiments demonstrate that the substance which acts on the heart is activated by cysteine. Control experiments showed that cysteine alone in a concentration of 1:1000 when administered twice to the same heart did not produce systolic contracture. Likewise, a solution containing heat-inactivated toxin plus cysteine failed to sensitize the heart to the action of SPA, and failed also to cause contracture of previously sensitized hearts.

3. Inhibition by Cholesterol.—As cholesterol is known to inactivate certain hemolytic and cardiotoxic agents of animal, plant, and bacterial origin, the effect of cholesterol on the cardiotoxic activity of the streptococcal preparation was investigated. The suspension of cholesterol was prepared according to the method used by Cohen, Shwachman, and Perkins (17). SPA was incubated with cholesterol 1:250,000 for 20 minutes in the cold prior to application to the heart. SPA treated in this way was found to be inactive as can be seen from the results in Table I.

4. Inhibition of Action of SPA by Sera Containing Antihemolysin.—The streptococcal preparation employed in this study contained a hemolysin of high potency. The hemolysin was markedly thermolabile, reversibly inactivated

by oxygen, susceptible to inhibition by cholesterol, and could be neutralized by antistreptococcal sera. These properties are in agreement with the findings of Smythe and Harris (18), Herbert and Todd (19), and others who have studied

TABLE I

Properties of Cardiotoxic Factor

Nature of experiment	Treatment of streptococcal preparation	Concentration of reagents during activation with cysteine		Concentration of streptococcal preparation applied to heart*	Effect of contracturing dose
		Streptococcal preparation	Cysteine		
Heat lability	Strep. prep. 1:10 heated for 10 min. at 60° C.	1:100	1:1000	1:250	No specific effect
	Unheated control	1:100	1:1000	1:250	Almost complete systolic contracture
Activation by cysteine	Bubbled with O_2 for 8 min.	No activation with cysteine		1:100	No specific effect
	Bubbled with O_2 for 8 min.	1:20	1:250	1:200	Complete systolic contracture
Inhibition by cholesterol	Strep. prep. 1:250 plus cysteine 1:2500 plus cholesterol 1:250,000 for 20 min. in cold.	1:100	1:1000	1:250	No specific effect
	Strep. prep. 1:250 plus cysteine 1:2500 for 20 min. in cold.	1:100	1:1000	1:250	Complete systolic contracture

* Two administrations.

the oxygen-labile hemolysin of the hemolytic streptococcus. The similarity of the properties of the cardiotoxic factor, described in the foregoing paragraphs, to those of the oxygen-labile hemolysin suggested that cardiotoxic and hemolytic actions may be functions of a single streptococcal product. With these considerations in mind, experiments were done for the purpose of finding out whether antihemolytic sera can inhibit the cardiotoxic action of SPA.

As has been emphasized previously, the application of SPA to the normal frog's heart does not result in systolic contracture but alters the state of the heart in such a way that systolic contracture occurs when SPA is applied a second time. In preliminary experiments it was found that the development of systolic contracture could be prevented by appropriate concentrations of antihemolytic sera. It was also found that the application to a normal heart of antihemolytic serum mixed with a sensitizing dose of SPA did not result in sensitization of the heart. These qualitative findings were extended to a roughly quantitative study in which the capacity of sera to neutralize the cardiotoxic action of SPA was compared to the antihemolytic capacity of the sera.

The capacity of a serum to inhibit systolic contracture was measured by mixing a series of dilutions of serum with a contracture-producing dose of activated SPA, and applying the mixtures, after 3 minutes at room temperature, to hearts which had previously been sensitized to SPA. For each serum, high concentrations inhibited systolic contracture while low concentrations failed to inhibit. Moreover, it was possible to find a concentration which partially inhibited contracture; this concentration was selected as the endpoint of the titration. For technical reasons, precision greater than that attained by employing a series of twofold dilutions of serum was not sought. The contracture-producing dose of SPA was the same in all the serum titrations and was equivalent in hemolytic units to twice the test dose employed in the titrations of antihemolytic action.

Eight specimens of horse serum were titrated for anticardiotoxic and antihemolytic potencies. The series included three specimens of normal horse serum, three specimens of antistreptococcal serum, and two of bivalent gas gangrene antitoxin. The antihemolytic potencies of the sera were measured by the method of Todd (20). The test dose of hemolysin was 0.00033 cc. or 20 of our hemolytic units. There was a thousandfold difference in antihemolytic potency between the strongest and the weakest serum. This difference, as well as intermediate differences in antihemolytic action of the other six sera, were found to be paralleled by the anticardiotoxic potencies (Table II).

The normal horse sera showed the lowest antihemolytic power and also the weakest anticardiotoxic action. The antistreptococcal sera and one of the specimens of gas gangrene antitoxin were the most potent inhibitors of hemolytic as well as of cardiotoxic action. Considering the error inherent in the technique employed, the agreement between the two properties measured is quite good,—actually better than was anticipated.

Owing to difference in endpoints employed in the titrations of antihemolytic and anticardiotoxic potencies, the volumes of serum stated in Table II are those corresponding to 100 per cent neutralization of hemolytic activity and to 50 per cent neutralization of cardiotoxic activity of the test doses employed. Since the test dose of toxin used in the titrations of cardiotoxic activity was twice that employed in the titrations of hemolytic activity, the volumes of serum representing the endpoints of the two titrations would be expected to be approximately the same. As can be seen in Table II, the two volumes were approximately equal. The marked anticardiotoxic property of one of the gas gangrene sera is notable inasmuch as the theta toxin of *Clostridium welchii*,

Type A, and the oxygen-labile hemolysin of *Streptococcus pyogenes* have been shown to possess similar properties and to be immunologically related (Todd (21)).

As the antiscarlatinal potencies of two of the antistreptococcal sera, Le47H445 and Le2745, were known, the capacity of these two sera to neutralize scarlatinal toxin on the one hand, and cardiotoxin on the other, could be compared. The ratio of anticardiotoxic power of the two sera was 4.7 while the ratio of antiscarlatinal power was 46, or approximately 10 times as great. The anticardiotoxic capacities of the two sera therefore did not parallel their capacity to neutralize scarlatinal toxin. The ratio of antihemolytic power, however, is 2.6, and this is of the same order as the anticardiotoxic ratio of the two sera.

TABLE II

Comparison of Antihemolytic and Anticardiotoxic Potencies of Various Specimens of Horse Serum

Serum	Amount of serum* required to neutralize hemolytic activity of SPA	Amount of serum required to partially inhibit cardiotoxic activity of SPA
	cc.	*cc.*
Normal horse serum No. 719	0.050	0.050
Normal horse serum No. 746	0.059	0.033
Normal horse serum No. 748	0.0034	0.0033
Antistreptococcal serum Le47H445‡	0.00024	0.00017
Antistreptococcal serum Le2745‡	0.00062	0.00080
Antistreptococcal serum BL	0.000050	0.000077
Gas gangrene antitoxin EL	0.00074	0.00062
Gas gangrene antitoxin Cu	0.0023	0.0017

* The reciprocal of these numbers equals the antistreptolysin-O titer in Todd units per cubic centimeter (20).

‡ Sera Le47H445 and Le2745 contained 6500 and 140 units per cc. of scarlet fever antitoxin, respectively. This information as well as the two sera were kindly supplied by Dr. J. N. Adams, Jr., Lederle Laboratories, Inc. We are indebted also to Dr. H. W. Lyall and Dr. E. Hazen, New York State Department of Health, to Dr. E. W. Todd, the Wellcome Laboratories, to Eli Lilly and Co., and to the Cutter Laboratories for other samples of sera.

DISCUSSION

The cardiotoxic factor, so far as it has been studied, possesses the following properties: It is a non-dialyzable, thermolabile product of *Streptococcus pyogenes* which is activated by cysteine and which is inhibited by cholesterol. Its action is inhibited by high concentrations of normal horse sera and by low concentrations of certain immune horse sera. The anticardiotoxic potencies of the sera studied were found to be directly proportional to their capacity to neutralize

oxygen-labile hemolysin of *Streptococcus pyogenes*. These findings strongly suggest that the cardiotoxic factor is closely related to, perhaps identical with, the oxygen-labile hemolysin of the streptococcus.

The pneumococcus, *Cl. welchii*, and *Cl. tetani* are known to produce oxygen-labile hemolysins which resemble closely that of the hemolytic streptococcus. It was therefore of interest to know whether the cardiotoxic factor is formed only by the streptococcus or by these bacteria as well. Although this point has not been thoroughly investigated, preliminary observations indicate that the cardiotoxic factor is not peculiar to the streptococcus alone. The hemolysin of a rough variant of pneumococcus, Type II, strain D39, prepared by the method of Cohen, Halbert, and Perkins (22) produced effects on the frog's heart which were indistinguishable from those of the streptococcal preparation. Likewise a preparation of *Cl. welchii* theta toxin,[2] which contained little or no alpha toxin, had an action on the heart similar to that of the streptococcal toxin. These results indicate that the sensitization described in this paper has bearing on the toxic action not only of the streptococcus but of other bacteria as well.

Such chemically diverse compounds as the cardiac glycosides and bile acids (23), the polypeptide tyrocidin (24), oxidizing agents (6, 25), and calcium ions are capable of producing systolic contracture of the isolated frog's heart. Although certain bacterial toxins, notably the toxin of *Cl. septicum* and one of the toxins, presumably alpha toxin, of *Cl. welchii* are known to induce systolic standstill of the frog's heart, their action takes place only after a latent period and the systolic standstill which develops is irreversible. The streptococcal toxin differs from most of the above substances in inducing a systolic contracture which is rapid in development and which is reversible. Its capacity to sensitize the heart to subsequent application of this toxin is an additional property which to the best of our knowledge is not possessed by other agents.

A phenomenon consisting in the sensitization of intestinal smooth muscle to cysteine by previous exposure of the tissue to this amino acid or to cystine was described by Voegtlin and Dyer (26). Since cysteine is one of the reagents employed in our experiments and since the sensitization which we have described bears a striking resemblance to that discovered by Voegtlin and Dyer, the possibility of a close relationship between the two phenomena was carefully considered. Experiments in which we attempted to produce systolic contracture of the heart by means of two applications of cysteine alone, or by sensitizing the heart with SPA and attempting to induce contracture with cysteine alone, and *vice versa*, were negative. In view of these results there does not seem to be more than a superficial similarity between our observations on the frog's heart and those of Voegtlin and Dyer on smooth muscle.

What relationship, if any, the sensitization described in this paper may have to the pathogenesis of streptococcal infections remains obscure. It should be

[2] Kindly supplied by Dr. Mark H. Adams.

emphasized that the effects of a toxin on an isolated organ need not be necessarily transferable to an intact organism, and that the observed response of the isolated frog's heart to a bacterial product may not have a direct counterpart in the mammalian heart. However, the fact that streptococcal hemolysin or a substance indistinguishable from it sensitizes the heart in the manner described and affects the contractile mechanism of the frog's heart has been clearly shown. It is thereby demonstrated that the action of this bacterial product is not limited to the lysis of blood cells.

SUMMARY

1. A study has been made of the effect on the isolated frog's heart of a preparation derived from the supernatant fluid of cultures of hemolytic streptococcus.

2. The streptococcal preparation was found to induce systolic contracture, the contracture usually developing only after the second of two administrations of the preparation. It was found that a single application of the streptococcal preparation sensitized the heart to a second application.

3. The properties of the cardiotoxic factor so far as they have been studied were found to be identical with those of the oxygen-labile hemolysin of streptococci. The capacity of normal and of immune sera to neutralize the cardiotoxic action paralleled the antihemolytic potency of the sera.

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