THE ANALYSIS OF STREPTOCOCCAL INFECTIONS

V. Cardiotoxicity of Streptolysin O for Rabbits in Vivo*

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Previous reports from this laboratory have pointed out the importance of agar precipitin technics as tools for the analysis of human streptococcal infections, as well as of infectious diseases generally (1–6). The use of human convalescent serum as antibody source enabled the estimation of the number of toxins or antigens released by the microorganism in the tissues during the course of the illness. The same system also enabled us to follow the purification of each of the antigens thus shown to be produced in vivo. Our studies have revealed that a very large number of streptococcal extracellular antigens were demonstrable with human sera or gamma globulin, but very few cellular products appeared to reach antibody-forming sites in significant amounts. For this reason, much of the recent effort in this laboratory has been devoted to the purification of the extracellular streptococcal components. Thus far, approximately nine of the twenty antigens detected with human gamma globulin have been purified to a greater or lesser degree.

The ultimate goal of these studies was to examine the pathogenetic and biological significance of each isolated antigen and to determine its identity and properties. The present report records the first attempt to assess the pathological significance of some of these components. Particular stress has been placed on streptolysin O because of its possible significance in the pathogenesis of rheumatic fever. Several of the other separated antigens have also been tested for their systemic effects.

Rheumatic fever is well known to be preceded by streptococcal infections, and strong evidence indicates that this microorganism causes the disease (7). The precise mechanism has never been established, however. It has been suggested that rheumatic fever may be the result of an unusual intoxication by some product or products of the streptococcus (8), or that hypersensitive (9), or autoimmune mechanisms (10) are operative. Whatever the mechanism, the specific cause and effect relationship between streptococcal infection and rheu-

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mictic fever makes it extremely likely that some particular substance of this microorganism is etiologically involved. Of the numerous known streptococcal toxins or antigens, circumstantial evidence has accumulated in the literature which is compatible with the speculation that streptolysin O may be a factor involved in the production of cardiac lesions. These lines of evidence are:

1. Bernheimer and Cantoni (11) first showed that the frog heart is very susceptible to this in vitro. Kellner, Bernheimer et al. (12) then demonstrated that the isolated rabbit heart is extremely sensitive to very small concentrations of this substance in vitro, when perfused through the coronary arteries. Under these circumstances, irreversible cardiac standstill occurred. These studies were carried out with concentrates of unknown complexity.

2. Schwab et al. (13) observed that rabbits pretreated with certain streptococcal factors developed a high incidence of focal cardiac lesions upon subsequent injection of streptolysin O containing culture filtrates. They presented evidence that streptolysin O was the substance causing the heart lesions.

3. Murphy and Swift (14) and others (15) have shown that repeated streptococcal infections of rabbits will cause focal cardiac lesions. Kirschner and Howie (16) confirmed these reports, and found a correlation between the incidence and severity of the heart lesions, and the intensity of rise of the antistreptolysin O titer of the serum. No such correlation was found with the antihyaluronidase responses in these rabbits.

4. Streptolysin O is almost uniformly produced in vivo during the course of human streptococcal disease, as evidenced by the very frequent rises of antistreptolysin (ASO) titer following infections (7).

5. Patients with rheumatic fever tend to have higher antistreptolysin O titers than patients with uncomplicated streptococcal disease (e.g., 7, 17, 18). They also tend to reveal higher titers of some other antistreptococcal antibodies (e.g., references 19, 20). In response to non-streptococcal antigens, however, rheumatic patients do not produce more antibody than non-rheumatic subjects (21–23). These data therefore suggest a more intense antigenic stimulus in rheumatic patients during the course of streptococcal infections, at least with streptolysin and several other secreted antigens.

It is perhaps of some significance that rheumatic fever recurrences can be prevented by curing streptococcal infections in the early stages with sufficient penicillin. Enough penicillin must be given so that the antibody responses (including ASO) will be stifled (24–26).

6. Coburn and Pauli (27) isolated 40 strains of hemolytic streptococci from cases of acute pharyngitis in 38 subjects with rheumatic fever. Infections by 20 of the strains resulted in rheumatic recurrences, while infections with the other 20 did not. These investigators reported that practically all strains from infections resulting in rheumatic fever produced large quantities of streptolysin O in vitro. Only 7 of the 20 non-effective streptococci were found to produce smaller quantities of this substance under the same growth conditions. Parallel results were obtained with reference to the synthesis of erythrogenic toxin.

7. Cholesterol is known to be a potent inhibitor of streptolysin O hemolysis (28). It has been shown that mice given injections of cholesterol (29), or detergents which will raise the blood cholesterol level (30) may be resistant to streptolysin O toxicity.
It has been stated (31) that rheumatic patients with myxedema, nephrosis, or diabetes mellitus seem refractory to rheumatic fever recurrences. Hypercholesterolemia is associated with these diseases. Patients with hyperthyroidism (and hypocolesterolemia) were reported to show increased susceptibility to rheumatic fever. Coburn (32) has reported that a diet producing hypercholesterolemia (egg yolk powder) will protect rheumatic subjects against rheumatic recurrences following streptococcal pharyngitis.

In view of the above cited investigations, it was felt of importance to examine the biological effects of the purified streptolysin O in vivo, with special reference to its cardiovascular effects. A preliminary description of these has been reported (5). The present study describes the detailed changes produced in rabbits in acute experiments with streptolysin O from 2 streptococcal strains belonging to different serological groups, A and C.

Materials and Methods

One lyophilized streptolysin O fraction was prepared from the Group A streptococcal strain C203S, by a combination of continuous flow electrophoresis and column chromatography reported previously (peak VII, Text-fig. 5 of reference 4). It revealed about 160,000 hemolytic units (HU)/mg. protein. Immunologically, it showed a predominant precipitin band with streptolysin specificity, which titrated to high dilutions (0.012 mg./ml.) using human gamma globulin. It was contaminated by two other antigens in small amounts (at 1 mg./ml. only). One of these was identified as diphosphopyridine nucleotidase (DPNase) immunologically, and the other is at present unidentified.

The streptolysin O of the Group C strain was similar to that reported previously (3), but had been made from a different batch of culture filtrate and was of somewhat lower potency. It contained about 60,000 HU/mg. protein, but also was contaminated by small amounts of DPNase. No other antigens but these were detectable with human gamma globulin. The Group C streptolysin in the lyophilized state contained about 40 per cent by weight of non-protein material as estimated by ultraviolet absorption measurements. The dry weight of the Group A product appeared to be all protein.

In all instances, the streptolysin was dissolved in 0.1 M sodium phosphate buffer at pH 7.2. Activation was carried out by adding l-cysteine-HCl as a neutralized solution in the same buffer to a final concentration of 10 mg./ml. After warming at 30°C. for 10 minutes, it was kept in an ice bath until use, never later than 3 hours after preparation.

The rabbits used in this study were albino of either sex, 2.6 to 3.2 kg. All were obtained from one breeder (Rockland Farms, New City). The injections were given intravenously into the marginal ear veins as rapidly as possible. Most of the rabbits received the same volumes of cysteine solution (10 mg./ml.) alone as a control, from 5 to 30 minutes prior to the administration of the streptolysin dose (0.2 to 2 ml.).

Other streptococcal fractions used included purified proteinase, prepared by cysteine treatment from precursor which had been 3 X recrystallized and 1 X rechromatographed (see reference 4). Also tested was a fraction rich in deoxyribonuclease B, an “antigen excess” fraction containing only two antigens, one possibly related to erythrogenic toxin, and a possible complex of protein and C carbohydrate. In addition, five rabbits were given lethal doses of Shigella paradysenteriae type III endotoxin, prepared by the method of Goebel et al. (33).

The cholesterol suspension was prepared according to the technic of Cohen et al. (34). Pooled human gamma globulin was obtained through the kindness of the American Red Cross.
CARDIOTOXICITY OF STREPTOLYSIN O

from E. R. Squibb and Sons (Rahway, New Jersey). Its antistreptococcal antibody content has been reported (2-4).

All rabbits were followed electrocardiographically, the electrodes being placed on the clean shaven limbs with pediatric size metal contacts, in most instances. All of the animals were unanesthetized and were usually tied prone on their abdomens. On most of the recordings, the amplitude was adjusted so that 1 mv. = 1.15 cm. and the paper speed was 2.4 cm./sec. Standard leads were tested, and those giving the optimal wave tracings were used. A high degree of variability was found between individual rabbits.

In a number of cases, right and left fronto-occipital and transfrontal electroencephalograms, electrocardiograms (lead 2), and femoral arterial blood pressures were simultaneously recorded with a Grass polygraph model 5 (Grass Company, Quincy, Massachusetts). The blood pressures were obtained by means of an indwelling catheter implanted under local anesthesia using a strain gauge manometer.

RESULTS

Following intravenous injection of a lethal dose of streptolysin O, the rabbits rapidly developed a series of motor convulsions, with the head in extreme extension. Respiratory arrest usually followed the convulsions, and in most instances, death occurred in less than 5 minutes.

The results of the toxicity assays are shown in Table I, which also records the time elapsed until the first convulsive seizure. It may be seen that the LD₅₀ of the Group A concentrate is about 0.15 mg. dry weight, as compared to 0.75 mg. of the Group C concentrate. This latter figure represented about 0.45 mg. protein, and the difference in lethal end-point correlated fairly well with the hemolytic potencies of the fractions. In addition, the clinical manifestations were precisely the same with both preparations. The sharpness of the end-point for both, and the similarity of the time required for convulsions are evident.

Electrocardiographic Effects of Large Lethal Doses of Streptolysin.—Profound electrocardiographic changes could be observed within 3 to 5 seconds following the injection of 3 to 6 multiples of the LD₅₀. Examples of these are shown in Text-figs. 1 and 2 for the Group A and Group C fractions, respectively. In three instances (Text-figs. 1a, 1b, 2a) there was an extremely rapid transition from a normal sinus rhythm to ventricular arrhythmia, followed by ventricular fibrillation and standstill. In Text-fig. 2b, a similar rapid transition from a normal sinus rhythm to ventricular arrhythmia occurred. Twenty-eight seconds after injection, temporary recovery apparently took place, with AV dissociation followed by sinus tachycardia. ST depression was still present, however, and 51 seconds after injection multifocal ventricular arrhythmia began, followed by standstill.

Electrocardiographic Effects of Smaller Lethal Doses.—When smaller, but still lethal doses of either streptolysin preparation was given (1 to 2 LD₅₀), the sequence of events was more prolonged. Examples of these are shown in Text-figs. 3 and 4 for the Group A and C fractions, respectively. It may be seen that
### Table I

**Toxicity for Rabbits of Intravenous Activated Streptolysin O from 2 Streptococcal Strains**

<table>
<thead>
<tr>
<th>Dose (dry wt.)*</th>
<th>Result</th>
<th>Time onset convulsions</th>
<th>Dose (dry wt.)</th>
<th>Result</th>
<th>Time onset convulsions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mg.</strong></td>
<td></td>
<td></td>
<td><strong>mg.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D</td>
<td>11 sec.</td>
<td>10</td>
<td>D</td>
<td>11 sec.</td>
</tr>
<tr>
<td>0.5</td>
<td>D</td>
<td>15 sec.</td>
<td>5</td>
<td>D</td>
<td>13 sec.</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>35 &quot;</td>
<td>D</td>
<td>23 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>65 &quot;</td>
<td>2.5</td>
<td>D</td>
<td>13 sec.</td>
</tr>
<tr>
<td>0.25</td>
<td>D</td>
<td>44 sec.</td>
<td>D</td>
<td>30 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>62 &quot;</td>
<td>D</td>
<td>75 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>65 &quot;</td>
<td>D</td>
<td>90 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>95 &quot;</td>
<td>1.5</td>
<td>D</td>
<td>16 sec.</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>105 &quot;</td>
<td>D</td>
<td>60 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>180 &quot;</td>
<td>2 hrs.‡</td>
<td>D</td>
<td>63 &quot;</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>2 hrs.‡</td>
<td>D</td>
<td>63 &quot;</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>D</td>
<td>75 min.</td>
<td>D</td>
<td>106 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>2 hrs.‡</td>
<td>1.25</td>
<td>D</td>
<td>56 sec.</td>
</tr>
<tr>
<td></td>
<td>S.§</td>
<td>24 &quot; ‡</td>
<td>D</td>
<td>60 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>65 &quot;</td>
<td>D</td>
<td>65 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>87 &quot;</td>
<td>D</td>
<td>90 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>129 &quot;</td>
<td>D</td>
<td>168 &quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>168 &quot;</td>
<td>1.0</td>
<td>D</td>
<td>36 sec.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>174 &quot;</td>
<td>D</td>
<td>45 min.</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>S</td>
<td>168 &quot;</td>
<td>0.75</td>
<td>D</td>
<td>32 sec.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>D</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>D</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5 min.</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The doses represent milligrams of lyophilized powder.
‡ Approximate times of death; convulsions may or may not have occurred.
§ D, died; S, survived at least 2 days.
CARDIOTOXICITY OF STREPTOLYSIN O

Text-Fig. 1. Extensive and electrocardiographic changes produced by 3 and 6 LDs of Group A streptolysin O in the resting active state intravenously. The time of injection is marked on the 1 second interval record. (a) 3 mg. (b) 6 mg. The lower strip starts 9 seconds after the injection was completed.
TEXT-FIG. 2. Extremely rapid electrocardiographic effects produced by 2 and 3 LD₅₀ doses of Group C streptolysin O. The follow-up strips represent sample recordings at the time intervals noted, following completion of the injections. (a) 2.5 mg. (b) 1.5 mg.
the changes were not always the same. Common alterations found were AV nodal rhythm, depression of the ST segment, and T wave elevation or depression, or biphasic T waves. Also seen were PQ and QRS prolongation, and decrease in the amplitude of the P waves and the QRS complex. Very temporary sinus bradycardia was frequently noted for several seconds following the injection. These changes were followed by ventricular extrasystoles, ventricular tachycardia, ventricular flutter, and fibrillation, with eventual standstill of the heart.

**Electrocardiographic Effects of Non-Lethal Doses.**—Of the thirteen rabbits listed in Table I which received non-lethal doses (1/2 to 2/3 LD₅₀) of the streptolysin preparations, often no changes in behavior were observed. Sometimes they appeared somewhat sluggish for a period of up to several hours, but this never seemed to be severe. Temporary electrocardiographic changes were found in nine of these animals, however. Examples of these temporary changes are found in Text-fig. 5. Sinus tachycardia (Text-fig. 5a), sinus arrhythmia (Text-fig. 5b), electrical alternans (Text-fig. 5c), diminution of the QRS amplitude (Text-fig. 5c), ST depression (Text-figs. 5b, c), negative or biphasic T waves (Text-figs. 5c, e), as well as ventricular extrasystoles (Text-fig. 5a) or unifocal ventricular premature beats (Text-fig. 5d) were present at times, but were reversible. The ST segment alterations might last only a few minutes (Text-fig. 5a) or for as long as several days (Text-fig. 5b). Runs of extrasystoles, as well as electrical alternans were seen in four of these animals. These changes lasted for 2 to 10 minutes. In three animals they began 5 to 10 minutes following the injection, but in one case, they were only detected at 18 hours (Text-fig. 5d).

**Controls.**—In order to ascertain that the toxic effects were due to the streptolysin itself, the following series of control studies were carried out.

Cysteine solution failed to cause any detectable changes whatsoever, even in the maximum doses used (20 mg./2ml.). For most of the streptolysin injections a volume of 0.5 to 1.0 ml. was used, containing only 5 to 10 mg. of cysteine. Cysteine control injections were given to almost all of the rabbits 5 to 30 minutes prior to the streptolysin dose. In the few which did not receive the preliminary cysteine control dose, the toxicity of the streptolysin itself was unaltered.

**Other Streptococcal Antigens and Shigella Endotoxin.**—Four other extracellular Group A streptococcal antigen fractions which had been separated electrophoretically and chromatographically in previous studies (4) were tested intravenously. Only limited numbers of animals could be examined because of the shortage of materials. None of these showed any significant electrocardiographic changes.

Two rabbits received 5 mg. each of a fraction containing only two detectable components, the “antigen excess” fractions. This material was similar to that
TEXT-FIG. 3. More prolonged duration of electrocardiographic effects with smaller lethal doses of reduced Group A streptolysin O.
Fig. 4. More prolonged duration of electrocardiographic effects with smaller lethal doses of reduced Group C streptolysin O.
found in chromatographic peak II of Text-fig. 5 in reference 4. It may be recalled that immunological evidence related one of those components to erythrocytic toxin. One animal was given this fraction after a solution of it was pre-treated with cysteine, as for streptolysin O. Although these two rabbits showed no detectable electrocardiographic changes up till 2 days, they died 5 and 6 days following the injections.

Two rabbits received 5 mg. each of a fraction rich in desoxyribonuclease B, but also containing some of the “antigen excess” components (peak III of Text-fig. 5 in reference 4). It is worth emphasizing that this fraction, which was
Text-Fig. 5. Temporary electrocardiographic effects produced by sublethal doses of reduced active Group A or Group C streptolysin O.
GROUP C STREPTOLYSIN O, REDUCED 0.75 MG.
LEAD 2

0 MIN.
5 MIN.
7 MIN.
127 MIN.

GROUP C STREPTOLYSIN O REDUCED 0.25 MG.
TEMPORARY ECG CHANGES

84 MIN. (AVR)
18 HRS. (AVR)
18.5 HRS. (AVR)

GROUP C STREPTOLYSIN O REDUCED 1.0 MG.
TEMPORARY ECG CHANGES (LEAD 2)

0 SEC.
26 SEC.
4 MIN.
devoid of hemolytic activity, was obtained from the same chromatographic run as that which yielded the Group A streptolysin O used here. The DNase B eluted in the step 0.01 M to 0.03 M phosphate, while the streptolysin eluted at 0.2 M and higher. One rabbit received the DNase B dose after pretreatment with cysteine. This animal survived, while the other died between 3 and 18 hours after the injection. Neither showed ECG changes, the survivor being followed for 6 days.

One rabbit received 5 mg. of an unadsorbed chromatographic fraction rich in an apparent complex of carbohydrate and protein (peak I of Text-fig. 4, reference 4). No definite electrocardiographic abnormalities were found, and the animal survived.

Three rabbits received 5 mg. of highly purified proteinase in 1 ml. This was activated from the precursor state by treatment with 10 mg. cysteine for 30 minutes at 30°C.; these conditions have been found adequate to produce transformation to the active enzyme. Interest in this substance was great because of the report by Kellner and Robertson (35) that it causes focal cardiac necrosis in rabbits, mice, and guinea pigs. One rabbit was seen to die 19 hours following the injection, about 5 minutes after the final electrocardiogram was recorded. No striking electrocardiographic abnormalities occurred during the postinjection period, although slight ST segment changes were seen terminally. Autopsy failed to reveal any gross lesions in the heart, or elsewhere. One other rabbit died between 5 and 18 hours, while the third survived. Neither showed any distinct ECG changes.

Five rabbits received varying lethal amounts of *Shigella paradysenteriae* type III endotoxin. The doses used ranged from 1.25 mg. to 10 mg., the time for death with the latter being 3 hours. Other tests indicated that the M.L.D. of this preparation was about 0.1 to 0.2 mg., so that the doses used above represented 10 to 100 M.L.D. No striking alterations of the electrocardiograms were noted, but it was of some interest that undulations of the recordings became quite extreme after about 1 hour due to accelerated respirations. The rabbit, whose ECG is shown in Text-fig. 6, received 5 mg. of endotoxin, and died between 3 and 15 hours following the injection.

State of Oxidation-Reduction of Streptolysin O.—It is well known that streptolysin O is a reversibly oxidizable substance, and that its hemolytic activity is evident only in the reduced state (36–38). In addition, during purification, it has been shown that streptolysin rapidly becomes reversibly oxidized to the extent of 95 per cent or more. The lyophilized preparations used here were also largely in this inactive configuration (about 95 per cent) and it was felt of importance to test their toxicity without cysteine reduction. The results of these tests are summarized in Table II, which clearly shows the much lower toxicity of these fractions when reversibly oxidized. Roughly 7 to 8 times more was required to kill than when in the reduced condition. These observations agree with those of Herbert and Todd (36) who reported limited data on
SHIGELLA PARADYSENTERIAE ENDOTOXIN 5 MG.

TABLE II
Toxicity for Rabbits of Reversibly Oxidized Streptolysin O

<table>
<thead>
<tr>
<th>Group A (LD₅₀ reduced = 0.15 mg.)</th>
<th>Group C (LD₅₀ reduced = 0.75 mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose of oxidized form (dry wt.)</td>
<td>Results</td>
</tr>
<tr>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>D</td>
</tr>
<tr>
<td>D</td>
<td>2 to 18 hrs.*</td>
</tr>
<tr>
<td>0.5</td>
<td>S</td>
</tr>
<tr>
<td>S</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>S</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>S</td>
</tr>
</tbody>
</table>

* Approximate time of death; convulsions may or may not have occurred.

the lethal toxicity of streptolysin O concentrates for mice in the oxidized and reduced state. The differences in lethal dose they found were of the same order of magnitude recorded here. Neither is quite as great as might be expected on the basis of the proportion of oxidized streptolysin, as evidenced by hemolytic titration. It seems likely, however, that some reduction takes place in vivo by the activity of blood or tissue cells. Evidence for this is furnished by observations of Pillemer and Ecker (39) showing that the sulfhydryl-dependent enzyme urease could be activated by the reducing potential of tissues.

It was of some importance that the injection of the oxidized streptolysin O
GROUP A STREPTOLYSIN O, REVERSIBLY OXIDIZED TEMPORARY ECG CHANGES (0.5 MG.) LEAD I

Text: Fig. 7. Electrocardiographic effects of reversibly oxidized streptolysin O.

0 MIN.
4.2 MIN.
6.0 MIN.
20.0 MIN.
18 HRS.
in lethal doses caused electrocardiographic changes similar to those seen with the activated fractions (see Text-figs. 7a and 7b). The time required for these changes to develop was longer than would be anticipated for the reduced form. In addition, several of the rabbits surviving the larger non-lethal doses of oxidized streptolysin O showed temporary ECG changes, such as the ventricular extrasystoles and bigeminal rhythm seen in Text-fig. 7c. In this latter rabbit, the extra beats began about 3.5 minutes following the injection, lasted for about 15 minutes, and then were only noted the next morning for a short while.

Effects of Cholesterol.—Further evidence that the toxic and lethal effects were due to streptolysin O was furnished by the tests with cholesterol, a well known potent inhibitor of this substance (11). Two rabbits received 0.5 mg. of the reduced Group A streptolysin (3 LD₉₀) that had been mixed with an aqueous 4 X crystallized cholesterol suspension and allowed to stand at room temperature for 5 minutes. One animal was given 2.5 mg. and the other 3.8 mg. of cholesterol in the mixture. Both survived, the former showing extrasystoles for a period of 2 minutes, starting 1½ minutes after the injection. A control rabbit given the same amount of this streptolysin solution, diluted with water and treated similarly, died with typical symptoms and electrocardiographic changes within 1 minute. Since diphosphopyridine nucleotidase has been shown to be unaffected by reducing agents, and by cholesterol (40), these data furnished evidence that this minor contaminant was not involved in the reactions seen.

Effect of Pooled Human Gamma Globulin.—Two rabbits were given intravenous injections of pooled normal human gamma globulin rich in antistreptolysin, one getting 2 ml. and the other 4 ml. (16 per cent solution). This was followed 5 minutes later by 1 M.L.D. of the Group C reduced streptolysin O (1.25 mg.). These animals were apparently completely protected against the lethal and cardiac effects of the streptolysin. The control rabbit receiving the same challenge without the human antibodies died typically within 2 minutes.

Four rabbits were given 1 M.L.D. of the reduced Group A streptolysin (0.25 mg.), 24 hours after receiving 4 ml. of the gamma globulin intraperitoneally. Three of these also received 1 ml. of the globulin intravenously 5 minutes prior to the challenge dose of streptolysin. Two of the latter survived, one with temporary ECG changes, while the other two died of streptolysin toxicity. Incidentally, in this series of experiments, two other rabbits were given 2 or 4 ml. of the gamma globulin intravenously, and showed temporary electrocardiographic abnormalities from this alone.

Blood Pressure and Electroencephalographic Data.—In eight rabbits given lethal doses of streptolysin O, simultaneous recordings were made of the femoral artery blood pressure, and in three of these, electroencephalograms (EEG) were also recorded. A typical experiment is shown in Text-fig. 8. The blood pressure decreased rapidly from 175/100 mm. mercury to 130/60 mm. after
Text-Fig. 8. Correlation of electrocardiographic, electroencephalographic, and blood pressure recordings in a rabbit given a lethal dose of Group C streptolysin O.
63 seconds, and then gradually to 25/25 mm. at 97 seconds. The electrocardiogram showed the same sequence of changes seen in Text-figs. 3 and 4, from sinus rhythm to ventricular tachycardia, ventricular fibrillation, and cardiac standstill. It is important to note that the electrocardiogram was significantly altered at the time of the first decrease in blood pressure. The inversion and widening of the QRS complexes at 54 seconds suggest that a ventricular conduction defect was already present. In other rabbits, the ECG changes were found after the blood pressure had begun to drop.

The electroencephalogram did not show patterns of convulsive discharges followed by EEG silence between the attacks of clonic and tonic motor convulsions. The lack of EEG changes strongly suggests that the central nervous system was not directly involved in the seizures begun by the streptolysin injections.

Histopathology.—Unfortunately, the animals studied above were not examined histologically. However, in some observations carried out earlier with less highly purified streptolysin O concentrates (2), a small number of rabbits, fifteen, were given single large sublethal or borderline lethal doses intravenously. The streptolysin O preparation (H96-13) was obtained by a modification of the method of Herbert and Todd (36), and contained approximately 16,000 hemolytic units/mg. of lyophilized powder. It also revealed at least four other antigens detectable with human gamma globulin. One of those was identified as proteinase precursor, but quantitative estimation with specific antiserum by Dr. S. Elliot showed it to be present only to the extent of 0.1 per cent. Other similar fractions, previously reported (2), but not used in these toxicity tests, contained 4 and 10 per cent of proteinase precursor.

In several animals sacrificed or dying from 24 to 30 hours after injection, small focal inflammatory lesions were found in the ventricles consisting almost solely of polymorphonuclear leukocyte accumulation about apparently damaged myofibrils (see Fig. 1a). After 2 days, the focal lesions were often more distinct, and the muscle cell damage more evident. The inflammatory cells were mixtures of polymorphonuclear leukocytes and round cells (see Fig. 1b, 1c). At this time, irregular cells with basophilic cytoplasm were commonly seen, and mitotic figures were not infrequent. At 5 or 6 days, focal healing lesions were noted in some instances (Fig. 1d), with an apparent loss of myofibrils. Control sections from a small group of rabbits receiving heat-inactivated streptolysin O concentrate failed to show similar lesions. The possibility that the lesions found were due to the proteinase precursor contaminant in these earlier streptolysin concentrates seems almost negligible. Rabbits receiving the reduced activated streptolysin were given a total of 0.6 mg. of lyophilized powder, of which roughly 0.1 per cent was proteinase. The dosage of this latter

* The authors are deeply grateful to Dr. S. Elliot for these estimations.
substance would therefore be about 0.0002 mg./kg., a quantity much lower than the 1.5 mg./kg. used by Kellner in his studies (35).

DISCUSSION

These observations demonstrate that streptolysin O can cause extraordinarily rapid effects on the cardiovascular system when given intravenously into rabbits in the reduced activated state. The finding that several multiples of the lethal dose can bring about almost complete electrical arrest of the heart in 3 to 4 seconds after completion of an injection taking 2 to 3 seconds, suggests that the toxic effects are felt almost as rapidly as the substance reaches the heart. The data accumulated in this study indicate that streptolysin O killed rabbits under these conditions by means of functional damage to the cardiac muscle. The electrocardiographic changes found were characterized by conduction defects and ventricular automatism. Similar changes have also been seen after toxic doses of digitalis glycosides (43). The above observations translate the in vitro data of Kellner et al. (12) to the in vivo environment. It would be of interest to determine whether the electrocardiographic effects of the in vitro perfusion system are similar to those found here. Such studies are planned.

The important question raised by such observations is whether they have any bearing on human rheumatic fever. The data described above all relate to acute experiments. In rheumatic fever, the disease pursues a rather insidious course. On the basis of the accumulated information, if one speculated that streptolysin O is the particular streptococcal product which causes the lesions of the human disease, account must be taken of three key characteristics of rheumatic fever.

1. The occurrence of a latent period of about 1 to 3 weeks following the streptococcal infection, after which frank symptoms of rheumatic fever appear.
2. The human illness follows a prolonged course of activity, long after the cessation of the initiating streptococcal infection.
3. The usual presence of appreciable to high titers of circulating antistreptolysin antibody.

A working hypothesis has been developed which incriminates streptolysin O and which accounts for all of these prime characteristics of the rheumatic state. It is postulated that during the streptococcal infection, an abundance of streptolysin O (and other extracellular products) are released into the tissues and the circulation. The secreted streptolysin combines with its antibody immediately, and circulates as an antigen-antibody complex. An equilibrium becomes established between continued secretion of streptolysin and further development of the anti-streptolysin (ASO), during the period of invasion by streptococci. Because of the well established, small, but definite dissociation of antigen-antibody complexes (44), the streptolysin-ASO complex acts as a source of slow release of active streptolysin. This substance, having a high degree of
predilection for certain tissues (e.g., heart, etc.), accumulates on or in the susceptible tissue cells until a toxic level is reached. At this point in time, the overt symptoms of rheumatic fever begin. Symptoms and damage will continue as long as significant amounts of streptolysin-antistreptolysin complexes are present to supply a source of streptolysin.

An experimental attempt to test this hypothesis could be undertaken. It should be possible to prepare activated streptolysin-ASO complexes in vitro with the highly purified substance and naturally occurring human antibody, or artificially developed rabbit antibodies. Injection of these complexes into rabbits might then be expected to result in cumulative toxic effects similar to those shown above, after an appreciable latent period. Electrocardiographic and histopathological studies over an extended period of time could be carried out. Control animals would receive non-streptococcal or other streptococcal antigen-antibody complexes.

It may also be possible to test this hypothesis to a limited extent by giving diets causing temporary hypercholesterolemia to active rheumatic patients. It would be necessary to ascertain that the cholesterol is free to react, as serum cholesterol is known to be often unavailable for combining with streptolysin (30, 41, 42). This naturally occurring inhibitor of streptolysin may adequately compete with the toxin, and prevent its combination with susceptible cells. Attempts to confirm Coburn’s prophylactic studies with diets causing hypercholesterolemia might also be in order. Another possible test of this hypothesis might be undertaken in patients during an acute attack of rheumatic fever. At this time, complete plasma exchange transfusion from a normal donor with low antistreptolysin titer might result in considerable elimination of the circulating streptolysin-ASO complexes. This should result in a rapid cessation of the disease activity.

It is worth pointing out again that the focal cardiac lesions Kirschner and Howie (16) produced in rabbits by repeated streptococcal infections appeared to be correlated with the antistreptolysin O titers, but not the antihyaluronidase responses. It is of interest that in the three detailed protocols recorded originally by Murphy and Swift (14), a marked rise in antistreptolysin O response was observed terminally. Coupled with the data reported above, it seems quite plausible that the focal cardiac lesions in rabbits, at least, are caused by streptolysin O.

These studies are an integral part of a series of investigations having as their goal the analysis of human streptococcal infections. The observations recorded in the present report thus represent the early efforts in characterizing the biological properties of these antigens. The four streptococcal antigen preparations tested here, other than streptolysin, failed to cause significant electrocardiographic effects, and were apparently much less toxic systemically. It is clear that far more extensive studies will be required, but the problem of ob-
taining larger quantities of the fractions must first be met. In addition, it is clear that future observations should preferably be carried out with the most highly purified fractions, which have met all the varied physical, chemical, and immunological criteria used at the present time for protein homogeneity.

SUMMARY

1. The rapid death which occurred after intravenous injection of activated streptolysin O from Group A or Group C streptococci was always preceded by profound electrocardiographic alterations. After several multiples of the LD₅₀ doses, cardiac electrical arrest or fibrillation could occur within 2 to 4 seconds after completion of the injection.

2. The streptolysin O preparations used were rather highly purified, but were known to be contaminated with small amounts of one or two other immunologically distinct components. Evidence that the observed results were due to streptolysin O was obtained by tests of the reversibly oxidized materials, and by cholesterol inactivation, as well as by in vivo protection with human gamma globulin rich in antistreptolysin antibodies.

3. Four non-streptolysin streptococcal antigens, partially or highly purified, failed to produce similar electrocardiographic changes, and were much less toxic. One of these was 3 X recrystallized and 1 X rechromatographed streptococcal proteinase. Shigella paradysenteriae type III endotoxin also did not produce striking electrocardiographic abnormalities.

4. A working hypothesis has been developed implicating streptolysin O as the etiological streptococcal factor responsible for the pathogenesis of rheumatic fever, which seems to account for the principal features of this illness.

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EXPLANATION OF PLATE 78

Fig. 1. Heart sections from rabbits receiving 0.6 mg. of reduced streptolysin O concentrate H96-13, prepared from the C203S strain. All were fixed in Bouin's fluid, and stained with hematoxylin and eosin, except 1c which was stained with Masson's trichrome stain.

Fig. 1 a. Rabbit 102-4, sacrificed at 24 hours. × 385. Focal acute inflammatory reaction subendocardially.

Fig. 1 b. Rabbit 101-6A, sacrificed at 2 days. × 190. Portion of a rather extensive lesion, right ventricle.

Fig. 1 c. Rabbit 101-4, sacrificed at 2 days. × 385. Higher magnification of a lesion similar to that seen in Fig. 1 b.

Fig. 1 d. Rabbit 102-5, sacrificed at 6 days, × 385. Scar-like lesion with a single muscle fiber in its center.
(Halbert et al: Cardiotoxicity of streptolysin O)