FURTHER STUDIES ON THE EFFECTS OF SPLEEN EXTRACT ON BACTERIA

LEO G. NUTINI, CORNELIUS W. KREKE, AND SR. M. PETRONELLA SCHROEDER

Institutum Divi Thomae, Cincinnati, Ohio

Received for publication February 21, 1945

In a previous paper (Nutini and Kreke, 1942) we have shown that extracts prepared from both human and beef spleens are capable of completely inhibiting the *in vitro* growth of *Streptococcus pyogenes* in concentrations as low as 1:2,000. Since the ultimate effect of any germicidal substance on an organism is usually preceded by changes in the metabolism, the present investigations were undertaken in an effort to determine whether such is the case with hemolytic streptococci subjected to the influence of spleen extract, and thus indirectly to determine the mode of action of the extract. For this purpose studies were conducted on the organism from both bacteriologic and chemical viewpoints, including morphology, staining reaction, fermentation, and biochemistry.

In addition, investigation of the effect of spleen extract on other organisms, reported in this same article (Nutini and Kreke, 1942) as preliminary work, has been greatly extended. The latter work was instituted with a view to determining whether or not the germicidal activity of the splenic extract was specific for hemolytic streptococci, since the preliminary tests seemed to indicate this. The experiments, performed in much the same manner as those with streptococci, were confined principally to staphylococci and organisms of the colon-typhoid-dysentery group, although the pneumococcus and tubercle bacillus were also included.

EXPERIMENTAL

In order to conduct experiments to determine the mode of action of the splenic extract on *Streptococcus pyogenes*, it was first necessary to arrive at a concentration of the material which would allow growth, even though retarded, in every instance of its use. In our previous work this concentration was found to be 0.05 per cent, but because we had completely used up the original extract, and because the proportion of the solid material in the extract to the amount of tissue extracted did not appear to be constant, it was thought advisable to determine concentrations for the new extract which would not kill the organism. Accordingly, a series of tubes containing brain heart infusion agar and varying concentrations of the extract, ranging from 0.05 to 1.0 per cent (0.05, 0.1, 0.2, 0.5, and 1.0), were inoculated with streptococci. In confirmation of the previous results with splenic material, growth occurred only in the tube containing 0.05 per cent of the extract, and this concentration was used throughout the following experiments.

The effect of the spleen extract on the staining reaction was the first of the bacteriologic reactions investigated. Control tubes containing brain heart in-

fusion broth and experimental tubes containing brain heart infusion broth and 0.05 per cent of the spleen extract were inoculated with *S. pyogenes* and allowed to incubate for 3 days. At the end of this time the organisms from the various tubes were transferred to separate slides and stained according to Gram's technique. Approximately 90 per cent of the cells grown under the influence of 0.05 per cent spleen extract were gram-negative, whereas those grown on the control plates stained 100 per cent gram-positive. The gram-negative organisms, however, did not differ materially from the gram-positive ones in their clumping, chain arrangement, or structure. From the alterations in the staining reaction we were led to suspect that the active factor in the spleen extract was not a nonspecific, general protoplasmic poison, but a substance which interfered specifically with the metabolism of the cell.

In an attempt to understand better this specific metabolic interference, both control organisms and organisms grown under the influence of 0.05 per cent spleen extract were subjected to a series of biochemical tests, using the ordinary bacteriologic techniques employed for the individual reactions (Topley and Wilson, 1937). Except for a slight retardation in the fermentation of lactose and galactose, the spleen extract had very little effect on the organism in regard to its carbohydrate metabolism. Although there were indications of other possible changes in the biochemical properties of the organism, these will not be discussed at the present time.

Having determined that the ultimate effect of spleen extract on *S. pyogenes*, namely, death, is preceded by certain changes in the metabolism of the organism, we next undertook experiments for the purpose of determining whether such action was specific for the streptococcus. Since *Staphylococcus* is related somewhat to *Streptococcus*, these experiments were first conducted on the former organisms. The techniques employed were similar in every detail to those used in the streptococcus experiments.

Fifteen petri dishes, divided into series of three, were prepared. Each series was made up of a single control dish, containing only nutrient agar, and two experimental dishes, in one of which 0.5 per cent spleen extract (pH 7.0) had been incorporated in the agar, in the other, 1.0 per cent of the extract. Five colonies of a two- to three-day-old culture of *Staphylococcus aureus*, isolated from the drainage of a moderately severe infection of the hand, were then inoculated into all the dishes with a fine platinum loop. At first there was apparent in all the experimental dishes a moderate depression in the colony growth of the organism as compared to that in the controls. After 3 days' incubation, however, actual stimulation was noted in the dishes containing 0.5 per cent spleen extract, and after 5 days, in the dishes containing 1.0 per cent of the extract. This increase in growth over the controls was manifested not only by an increase in diameter of the colonies, but also by an increase in their density. The increase by surface spread was determined by tracing the individual colonies and measuring the surface area of the tracings with a planimeter. Figure 1 illustrates both the inhibitory and stimulatory effect of the extract on the organism as determined by the planimetric measurements.

Coincident with or just prior to the time that the colony growth of the organisms in the experimental dishes was stimulated, a very interesting change in these colonies was noted. Although the original colonies implanted were uniformly orange in color, and although the control colonies continued to remain

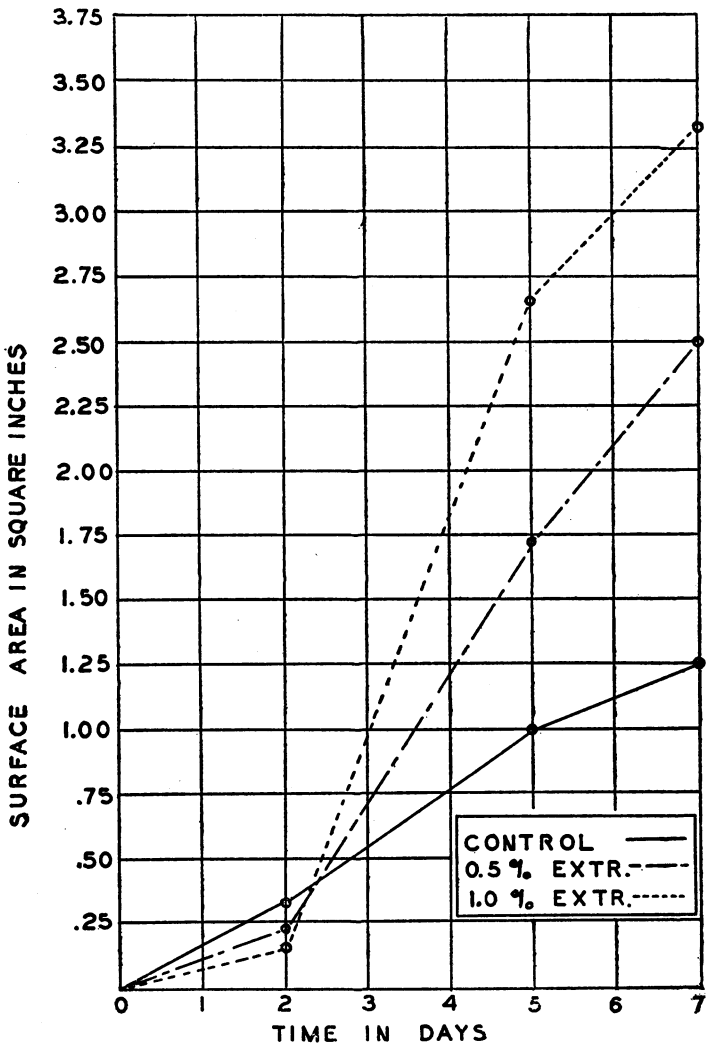

FIG. 1. EFFECT OF SPLEEN EXTRACT ON THE COLONY GROWTH OF STAPHYLOCOCCUS AUREUS

uniformly orange throughout the experiment, V-shaped wedges of white growth made their appearance in the experimental colonies at this time, gradually increased in size, and by the end of the sixth day constituted almost 100 per cent of the growth. To eliminate the possibility of any white contaminant in the original culture, organisms from the control dishes were passed through broth

and plated out a number of times, a procedure which resulted in the production of nothing other than orange colonies, and which thus verified the purity of the culture. This orange organism was then inoculated into a series of petri dishes prepared as in the preceding experiment. The results were identical with those secured for the original orange organism—namely, initial depression of growth followed by stimulation and the appearance of white organisms in the dishes containing spleen extract.

The change from an orange to a white organism was at first thought to be an adjustment of the organism to its environment; the orange organism, being depressed in growth, reverted to the white, which was stimulated. To satisfy this explanation, however, white organisms if planted on media containing spleen extract would necessarily have had to respond by immediate acceleration in growth, since they had already been altered to cope with the depressing effect of this material. To test this theory, therefore, the white organism was subjected to an experiment similar in every respect to that for the orange. Instead of the expected immediate stimulation, there occurred an initial depression in growth of even greater degree and duration than that for the orange, followed by slight stimulation of about the same degree. Consequently it cannot be assumed that the reversion of orange to white is a natural reaction to environment, but must rather be considered a change forced on the organism by the action of the spleen extract.

From the preceding experiments, therefore, it must be concluded that the action of the spleen extract in the case of *S. aureus* is twofold, affecting first a change in its colony growth with initial depression and subsequent stimulation, and second a variation in its colony morphology from an orange to a white form. This variation in colony morphology, however, is not to be regarded as a distinct genetic change, for on a single passage of the white organisms through brain heart infusion broth, followed by plating on nutrient agar, a reversion to the orange was always secured. Moreover, from our own observations and those of Bigger, Boland, and O'Meara (1927) this variation from orange to white is a common result with old cultures and may be produced as well by the simple addition of salt (Hoffstadt and Youmans, 1932) or some antiseptic dye to the medium. It is interesting to note, however, that after numerous subcultures under the influence of spleen extract, a white organism was secured which was apparently stable and did not revert to the orange when transferred to nutrient agar.

As in the case of the streptococcus, in which morphologic and perhaps biochemical changes took place before actual killing, it was thought that such changes might also occur in the conversion of orange to white *S. aureus*. Accordingly, morphology studies were undertaken. The results of these studies indicated that if such a change does take place in the staphylococcus it is unlike that for the streptococcus, for both white and orange organisms stain uniformly gram-positive and possess normal clumping. The only difference appears to be an increase in the viscosity of the white organisms.

Continuing further along this line, a series of fermentations and biochemical reactions were run both on staphylococci influenced by spleen extract and on

those grown normally. In table 1 are shown the results of this experiment. Here again, as in the case of the streptococci, there was no difference in the fer-

TABLE 1

Comparison of fermentations and biochemical reactions of staphylococci grown normally and those grown under the influence of 0.5 per cent spleen extract

FERMENTATIONS	1-DAY INCUBATION	4-DAY INCUBATION	5-DAY INCUBATION
Staphylococcus aureus grown normally			
Sugars			
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Galactose	+	+	+
Mannose	+	+	+
Inulin	−	−	−
Dulcitol	−	−	−
Salicin	−	−	−

BIOCHEMICAL REACTIONS	5-DAY INCUBATION
Tests	
Methyl red test	+
Voges-Proskauer reaction	−
Nitrate reduction	+
Gelatin liquefaction	+ (?)
Indole production	−

FERMENTATIONS	1-DAY INCUBATION	4-DAY INCUBATION	5-DAY INCUBATION
Staphylococcus aureus grown under influence of 0.5 per cent spleen extract			
Sugars			
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Galactose	+	+	+
Mannose	+	+	+
Inulin	−	−	−
Dulcitol	−	−	−
Salicin	−	−	−

BIOCHEMICAL REACTIONS	5-DAY INCUBATION
Tests	
Methyl red test	+
Voges-Proskauer reaction	−
Nitrate reduction	−
Gelatin liquefaction	−
Indole production	−

mentation reactions except for a possible slight slowing up of the process with those organisms grown in the presence of the extract. An important change

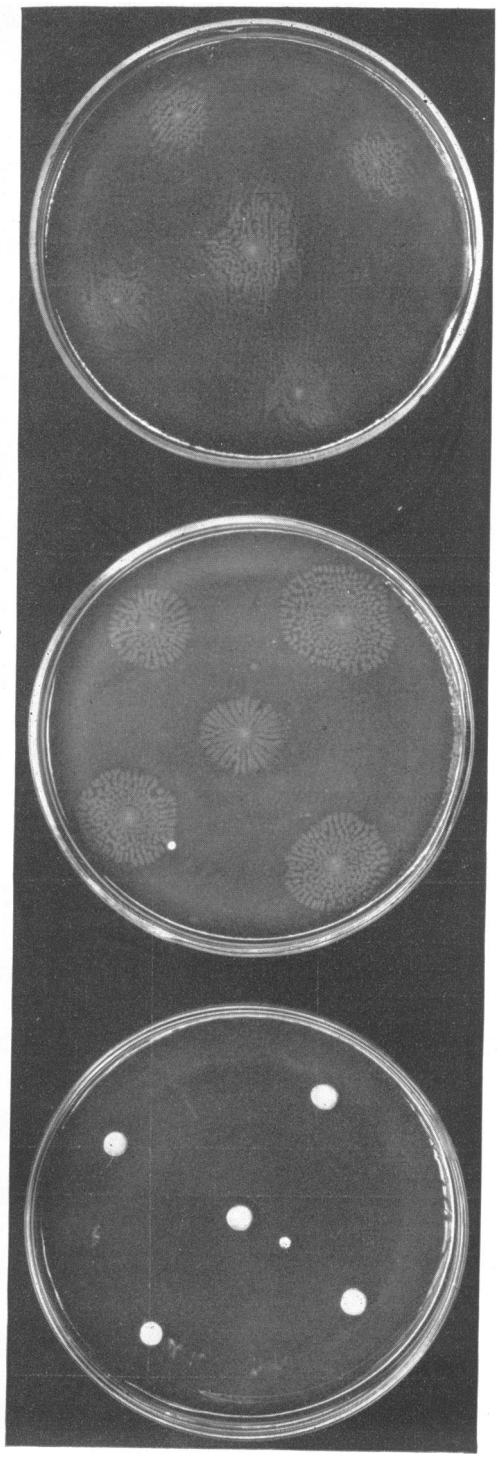

FIG. 2. ESCHERICHIA COLI
(Left) Control—2 days. (Center) 0.5% spleen extract no. 4. (Right) 1.0% spleen extract no. 4

took place in the biochemical reactions in that those organisms grown under the influence of spleen extract appeared to reduce nitrites to ammonia.

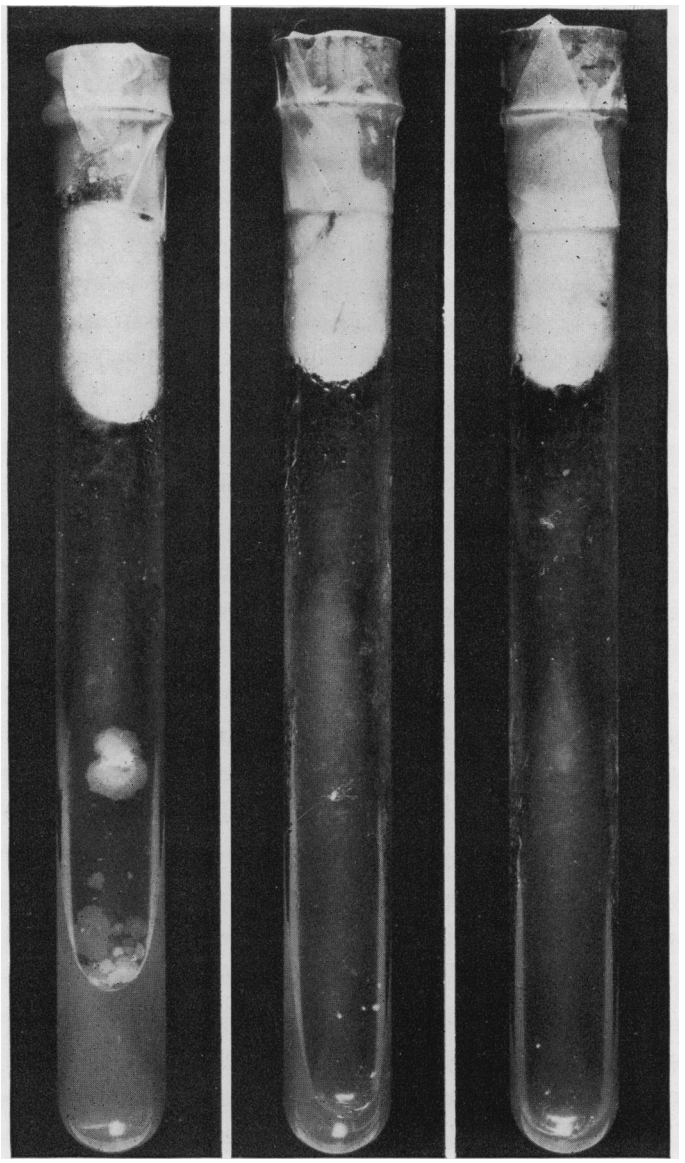

FIG. 3. TUBERCLE BACILLUS

(Left) Control—68 days on 6% glycerol agar. (Center) 0.5% spleen extract no. 1-A in glycerol agar. (Right) 1.0% spleen extract no. 1-A in glycerol agar.

In addition to streptococci and staphylococci, the effect of the spleen extract was studied on the pneumococcus, the tubercle bacillus, and a representative number of organisms of the colon-typhoid-dysentery group. The investigations

on the latter organisms were not nearly so extensive as those for the former and dealt solely with the morphologic aspect of the problem, the experimental procedures paralleling in every respect those for the streptococcus and staphylococcus.

Pneumococcus, type VII, isolated from a case of lobar pneumonia, suffered no apparent change when cultured in media containing as high as 1.0 per cent spleen extract. The organisms of the colon-typhoid-dysentery group, *Escherichia coli*, *Aerobacter aerogenes*, *Aerobacter cloacae*, *Proteus vulgaris*, *Salmonella paratyphi* A, *Salmonella paratyphi* (Children's), *Salmonella enteritidis*, *Eberthella typhosa*, *Shigella dysenteriae*, *Shigella paradysenteriae* (Flexner), *Shigella paradysenteriae* (Sonne), and *Alcaligenes faecalis*, on the other hand, responded by increased growth and changes in size and shape of the organisms. In the case of *E. coli* this increase in growth was also accompanied by the formation of arborescent colonies (figure 2). The experimental organisms are in general about twice the size of the controls and slightly plumper. The tubercle bacillus (isolated from a case of tuberculous meningitis and cultured on 6 per cent glycerol agar) appears to be the only other organism thus far studied which resembles the streptococcus in its response to the extract. This organism is definitely inhibited in its growth (figure 3), if not actually killed, in 0.5 per cent concentrations of the extract. We purposely limit ourselves at this time to mere inhibition rather than killing because, although our experimental cultures show no evidence of growth at the end of 60 days of incubation, the original planted material is still evident on the medium and might grow if transferred to a control plate. This will be investigated in future work.

DISCUSSION

In the present paper we have shown that spleen extract is not specific for *Streptococcus pyogenes*, but merely specific for this organism with respect to its germicidal powers. The mechanism of the action of the extract appears to be an interference in the metabolism of the cell, which in the case of streptococci is evidenced by direct killing in high concentrations and reversion of the gram-staining reaction, together with possible biochemical changes in low concentrations, and in the staphylococci by initial depression followed by stimulation and the production of white organisms. As the literature (Bartholomew and Umbreit, 1944) suggests, the change in the staining reaction of the streptococci might be due to the "stripping off" of the capsule of the organisms by the extract, since the ribonucleic acid in this layer is said to be the important factor in the gram-positive character of organisms. This undoubtedly is not the sole factor, however, since a similar change was not effected in gram-positive staphylococci subjected to the extract.

The literature (Bigger, Boland, and O'Meara, 1927; Hoffstadt and Youmans, 1932) also suggests that the white *Staphylococcus aureus* produced by the action of the spleen extract represents a rough strain or one of low virulence. By means of frequent cultures and subcultures we have been able to secure a stable white strain, and at present we are conducting animal experiments in an attempt to determine whether there is any difference in the virulence of the two organisms.

Animal experiments are also being conducted to determine whether the reversion of orange to white takes place *in vivo* under the influence of the extract. Preliminary animal experiments indicate that both of these effects exist and that staphylococcus infections can be controlled in animals by means of tissue extracts (Nutini and Lynch, 1945).

SUMMARY

Although spleen extract in concentrations of 0.05 per cent allows growth of *Streptococcus pyogenes*, it produces morphologic differences and perhaps changes in the biochemistry of the organism. In higher concentrations it is germicidal to the organism.

Extracts from human and beef spleens, even in high concentration, have no germicidal effect on *Staphylococcus aureus*. Spleen extract, after initially depressing the growth of *S. aureus*, later stimulates it. Along with this stimulation the original orange organism is transformed to a white type. The white organism is unstable and remains white only after frequent cultures and subcultures in media containing the extract.

The growth of pneumococci is unaffected by the spleen extract.

The colon-typhoid-dysentery group reacts to the spleen extract with increased growth and changes in the size and shape of the organisms.

The growth of the tubercle bacillus is inhibited by spleen extract.

REFERENCES

BARTHOLOMEW, J. W., AND UMBREIT, W. W. 1944 Ribonucleic acid and the gram stain. J. Bact., **48**, 567–578.

BIGGER, J. W., BOLAND, C. R., AND O'MEARA, R. A. Q. 1927 Variant colonies of *Staphylococcus aureus*. J. Path. Bact., **30**, 261–270.

HOFFSTADT, R. E., AND YOUMANS, G. P. 1932 *Staphylococcus aureus*. Dissociation and its relation to infection and to immunity. J. Infectious Diseases, **51**, 216–242.

NUTINI, L. G., AND KREKE, C. W. 1942 The toxic effects of splenic extracts on *Streptococcus hemolyticus*. J. Bact., **44**, 661–666.

NUTINI, L. G., AND LYNCH, E. M. 1945 Tissue extracts in controlling *Staphylococcus aureus* infections. Nature. In press.

TOPLEY, W. W. C., AND WILSON, G. S. 1937 The principles of bacteriology and immunity. Wm. Wood & Co., Baltimore.