EFFECT OF SULFANILAMIDE INJECTED SUBCUTANEOUSLY INTO RABBITS UPON HEMOLYTIC STREPTOCOCCI CONTAINED IN COLLODION SACS IMPLANTED INTRAPERITONEALLY[1]

ALBERT H. HARRIS AND JOHN K. MILLER

Division of Laboratories and Research, New York State Department of Health, Albany

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Experiments were undertaken to determine the effect of sulfanilamide upon hemolytic streptococci growing within the animal body, yet protected by collodion membranes from phagocytosis. The method of manufacturing the collodion sacs used in the experiments has previously been described (Harris, 1939). Theories regarding the rôle of phagocytosis in the drug's action have been amply discussed by Long and Bliss (1939). The following experiments, of which a preliminary report has been presented (1940), were undertaken to test the prevalent hypothesis that phagocytosis is essential to the effective bactericidal action of the drug *in vivo*.

MATERIAL AND METHODS

Sacs with membranes of varying porosities were made,[2] employing 7.9, 9.5, and 10.3 per cent "Parlodion" (Mallinckrodt) in glacial acetic acid. Each sac held approximately 3 ml. of fluid.

A virulent strain (No. 196) of hemolytic streptococcus originally isolated by Dochez from a patient with scarlet fever, and maintained in this laboratory since 1926 by weekly mouse passage, was used in these experiments. The collodion sacs were filled

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[2] Charles A. Clark prepared the sacs and took part in all of the experiments.

with varying numbers of microörganisms obtained from a 14- to 16-hour broth culture and suspended in a dialyzed peptone solution, which has been described by Brown and Robinson (1938). In this special medium (which fails to give a Heller reaction), hemolytic streptococci maintain their viability for several hours, yet do not multiply demonstrably.

A precipitating serum against rabbit serum was prepared by injecting undiluted rabbit serum into the wing vein of a White Leghorn hen on alternate days for three doses, the amounts injected being 0.5, 1.0 and 1.5 ml. The precipitating titer against rabbit serum was maintained by injecting from time to time 0.2 to 0.4 ml. of a 2 per cent solution of rabbit serum, following the recommendations of Wolfe (1936). The hen serum thus prepared yielded a precipitation ring when overlaid with rabbit serum, diluted as high as 1:1,000,000, after remaining one hour at room temperature.

The sac membranes of 7.9 per cent collodion within the peritoneal cavities of rabbits were permeable to substances that gave a precipitate with serum of the hen immunized against rabbit serum, and yielded a positive Heller reaction. Frequently they allowed streptococci to grow through, yet they invariably excluded leucocytes; nor was there evidence of fibrin formation within any of the sacs except on one occasion when a membrane became defective and the sac was found to contain both fibrin and leucocytes. With the 9.5 and 10.3 per cent membranes, the tendency of the streptococci to grow through the pores of the membranes was much less; the sac contents often either did not yield a precipitate with the serum of the immunized hen, or failed to give a positive Heller reaction; equally often, both reactions were negative. However, the presence or absence of specifically precipitable or coagulable substances had no demonstrable influence on the growth or destruction of the microörganisms. The tendency of microörganisms to grow through the pores of acetic acid collodion sacs of relatively high permeability, but not through the pores of alcohol-ether collodion membranes having comparable filtration speed is due, according to Asheshov (1933), to the fact that while the pores in acetic acid collodion

membranes are fewer in number, they are larger in size than those of alcohol-ether membranes. Hence, they may allow the passage of bacteria which the smaller pores of corresponding alcohol-ether membranes uniformly exclude.

It is important to bear in mind that one of the fundamental differences between *in vitro* work and experiments involving the *in vivo* use of collodion sacs lies in the fact that in the latter case there is a continual diffusion into the sacs of nutrient material and a diffusion out, of catabolic products. Thus, the microörganisms contained therein are inexhaustibly supplied with growth-promoting elements, while at the same time the deleterious effect of a changing pH and the accumulation of the dialyzable toxic products is avoided. Even a microörganism as delicate as the gonococcus was shown (1939) to survive for thirty-eight days under such circumstances.

For certain of the experiments, so-called marsupialized sacs were prepared. Short, glass-tipped tubes of soft rubber were sterilized by autoclaving and were attached to the glass tubes of the collodion sacs, after the latter had been filled with suspensions of microörganisms. Great care was exercised in this procedure to prevent contamination of the sac contents. After the rubber tubes were attached, the glass tip and the connections between glass and rubber were covered with heavy alcohol-ether collodion, and allowed to dry in air. A schematic diagram of this type of sac is shown at the top of plate 1. In contradistinction to the marsupialized sacs, those which were completely enclosed within the peritoneal cavities have been designated buried sacs.

IN VITRO EXPERIMENTS

Experiments were undertaken in test tubes for the purpose of controlling the subsequent animal experimentation. The sacs were treated in exactly the same way as in the case of the animal experiments except that instead of being placed in the peritoneal cavities of rabbits they were dropped into test tubes of beef infusion broth, to which in some instances 10 mgm. per cent sulfanilamide had been added. Bactericidal activity of the drug

was pronounced at from 39° to 40°C., but was absent at from 36° to 38°C. The growth was marked in sacs incubated in each temperature range in the absence of the drug, although it was slightly heavier in those incubated at the lower temperature. These findings confirm the observations of White and Parker (1938, 1939) that temperature is a factor of the highest importance in the action of sulfanilamide.

When the drug was added to the broth eight hours after the sac containing streptococci had been introduced, it evidenced only a bacteriostatic or relatively slight bactericidal activity, even though the temperature of incubation was 39.6°C.

IN VIVO EXPERIMENTS

Operative procedures

Rabbits weighing between 3 and 5 kgm. were used in the *in vivo* experiments. The fur was clipped from the abdomen and the skin prepared by washing with soap and water followed by iodine and alcohol. The animal was anaesthetized with ether. The peritoneal cavity was opened by a mid-line abdominal incision, the sacs were distributed to different parts of the peritoneal cavity, and the peritoneum and muscle layers were closed by a continuous suture of 00 plain catgut. The edges of the skin incision were approximated by a running mattress suture of black silk. The incision was covered by sterile cotton fixed in place by flexible collodion.

In introducing a marsupialized sac, the intestines were retracted away from the abdominal wall, an incision approximately 1 cm. was made through the skin, abdominal muscles, and peritoneum, and the tip of the marsupialized sac pulled out through this incision. In this manner the sac itself was carried into the abdominal cavity through the mid-line incision and allowed to remain horizontally across the abdominal cavity. The button-hole incision was closed around the tube by means of a purse string suture, and the mid-line incision was closed in the usual manner. Periodic aspiration of the sac contents was accomplished after the collodion on the tip of the external glass tube was destroyed in a match flame, by means of a long,

fine trochar and tuberculin syringe. The tip was closed after each aspiration by the application of alcohol-ether collodion.

EXPERIMENTATION

Experiment 1. A typical experiment follows, in which the sacs were not marsupialized, but were wholly buried within the peritoneal cavities of rabbits: a rabbit weighing 3,700 grams was given a subcutaneous injection of 20 ml. of 2 per cent sulfanilamide (400 mgm.) every four hours for forty-eight hours; the first dose was given two hours preoperatively. At operation, two

TABLE 1

Experiment 1. Temperature chart

HOUR POST-OPERATIVELY	TREATED RABBIT			UNTREATED RABBIT		
	Temperature	White blood cells	Polymorphonuclears	Temperature	White blood cells	Polymorphonuclears
	°C.			°C.		
1	38.3	9,800	46	38.3	10,200	44
3	40	13,000	79	40	9,400	58
7	40.1			40.2		
11	40.2			40		
15	40			40.1		
19	39.8	19,200	68	40	8,800	55
23	39.8			39.9		
27	39.5	9,600	67	39.8	13,200	48
31	39.4			39.8		
35	39.2			39.1		
39	39			39.1		
43	39	9,000	58	39.1	14,000	60

7.9 per cent collodion sacs were implanted in the peritoneal cavity, one containing a suspension of 750 streptococci per milliliter and the other 75,000 per milliliter. A second rabbit, weighing 4,330 grams, received two similar sacs but remained untreated. For the purpose of comparison, the assumption was made throughout these experiments that one colony on a poured plate represented one microörganism. The temperature was taken and white blood counts were made periodically during the test period. (See table 1.) At the end of forty-eight hours, both animals were sacrificed and autopsied. There was no

evident difference in the amount of reaction around the sacs in each rabbit. It was characterized mainly by a moderate amount of fibrin deposition, with enmeshed polymorphonuclear and mononuclear leucocytes. The concentration of the microörganisms and of the drug within the sacs was determined. (See table 2.) The latter determinations were made in the group of Mr. Leonard Hyman. Films prepared from the sediment of the contents of each sac after centrifugalization revealed no blood cells nor fibrin. The supernatant fluid in each instance yielded a positive reaction with the serum of the hen that was immunized against rabbit serum and with nitric acid.

Experiment 2. A similar experiment was performed using 9.5 per cent collodion sacs; the number of sacs used was doubled.

TABLE 2

Experiment 1. Colony counts on poured plate

Number of streptococci per milliliter

	TREATED RABBIT			UNTREATED RABBIT	
	Sac 1	Sac 2	Blood	Sac 1	Sac 2
Beginning of experiment....	750	75,000		750	75,000
Conclusion of experiment....	0	46		224,000,000	175,000,000
Sulfanilamide (milligrams per cent).....	17.6	24.2	26.6		

Dilutions containing 500 and 50,000 microörganisms per milliliter were employed. All of the sacs in the treated animal yielded fluid free of streptococci at the end of the experiment, whereas the sacs in the untreated rabbit yielded a heavy suspension of the microörganisms. The fluid in one of the sacs from the treated rabbit had become contaminated with staphylococci, which had multiplied to 80,000,000 per milliliter, apparently indicating that sulfanilamide had had no effect on them.

Experiment 3. In an experiment using 10.3 per cent collodion sacs, similar findings obtained. The contents of all of the sacs from the rabbit that had been treated were sterile. The average concentration of the free drug in the sacs was 7.8 mgm. per cent, and in the blood, 10 mgm. per cent. The differences in

the concentration of free sulfanilamide at the end of the various experiments were probably due to variations in the rate of absorption, excretion, and acetylation of the drug by the different rabbits.

Experiment 4. An experiment was undertaken with 10.3 per cent collodion sacs to determine whether or not streptococci could be destroyed when treatment of the experimental animal was begun during the period of rapid proliferation of the microorganisms. Accordingly, treatment was withheld for eight hours postoperatively. Four hours after the first injection of the drug, several million streptococci per milliliter were present, as estimated from the plating of 0.1 ml. samples from marsupialized sacs. Within eight hours after treatment was started, the number of microörganisms had commenced to decline, the drop being rapid at first and more gradual toward the end of the 48-hour test period. With the beginning of the decline, bizarre forms began to appear, similar to those described by Lockwood (1938); many cocci were swollen, and in several instances a number of cocci in a chain failed entirely to be stained by Gram's method. At the end of the 48-hour test period, the two buried sacs that originally contained a suspension of 200 and 20,000 streptococci per milliliter, respectively, were found to harbor only 400 and 420 viable microörganisms per milliliter. In view of a subsequent experiment (no. 7), there is reason to believe that the remaining streptococci would have been killed if therapy had been prolonged. The concentration of free sulfanilamide was comparatively high at the end of this experiment, being 33.5 mgm. per cent in the blood and 32.1 and 38.0 mgm. per cent in the two buried sacs.

Experiment 5. One could not be sure that the apparent killing effect of sulfanilamide represented actual destruction of the streptococci rather than merely complete inhibition of growth. It seemed possible that the presence of even small amounts of sulfanilamide transferred with the microörganisms to the poured plates might have effected permanent bacteriostasis. To obtain information on this point, streptococci were introduced into a sac of 10.3 per cent collodion in numbers that had in previous

experiments been killed or inactivated by sulfanilamide during the usual 48-hour test period. Into a second sac was introduced a quantity of streptococci larger than the maximum number that had usually been killed by injection of sulfanilamide, within the test period. Both sacs were implanted in a rabbit, and the usual course of injections was commenced at once. After forty-eight hours of therapy, the concentration of free sulfanilamide in the blood was 8.66 mgm. per cent. The sacs were not removed at this point but the administration of the drug was discontinued. The amount of free sulfanilamide in the blood dropped rapidly and none was detected in a sample taken forty-eight hours later. Seventy-two hours after this blood sample was taken, the sacs were removed and opened. In the sac that originally contained 1,260 streptococci per milliliter, the fluid was crystal clear and sterile. The concentration of free sulfanilamide in this sac was less than 0.01 mgm. per cent. A mouse inoculated with 0.5 ml. of the material survived. In the sac that originally contained 126,000 cocci, the fluid was milky in appearance and the number of microörganisms had increased to 104,000,000. The free sulfanilamide concentration was 0.05 mgm. per cent. A mouse injected with 0.5 ml. of the suspension died in less than twenty-four hours. The contents of neither sac yielded a precipitate with the serum of the hen that was immunized against rabbit serum, and the Heller test was also negative in both instances. These findings indicate that sulfanilamide is lethal to streptococci under the conditions of the experiment described, but that if the microörganisms are not entirely destroyed, they may proliferate rapidly when the effect of the sulfanilamide is removed.

Experiment 6. Among repeated experiments with different rabbits, two animals developed a slowly progressive fall in temperature, in one instance ranging between 37.7° and 36.6°C. for twenty-four hours, due we believe, either to an unfavorable reaction to the operative procedures, or, more likely, to the toxic effect of the drug. Through the aspiration and plating of measured amounts of marsupialized sac contents at regular intervals, it was apparent that the numbers of streptococci were not de-

clining at the rapid rate that had been observed in experiments where the rabbits' temperatures had remained at the normal level of between 39° and 40°C. Indeed, when one of the animals died after thirty-six hours of treatment, only one of the buried sacs yielded less than 90,000,000 streptococci per milliliter; this in spite of the fact that the average free sulfanilamide in the sacs at the time of death was 26.01 mgm. per cent. While it may be hazardous to draw conclusions from an animal experiment conducted under conditions of marked physiologic disturbance ending in death, it seems probable that the failure of sulfanilamide to exert a bactericidal effect was due, at least in part, to the temperature, which was far below normal for rabbits, and in the range wherein sulfanilamide is known to have relatively little effect *in vitro*.

Experiment 7. A final experiment was undertaken that combined various features of the preceding experiments, and was designed to determine whether or not very large numbers of hemolytic streptococci could be destroyed if therapy was initiated during the phase of active growth, and then prolonged over a period of three days. Accordingly, two rabbits weighing 3,200 and 4,100 grams, respectively, were prepared, and two sacs were marsupialized and two were buried in the peritoneal cavities of each. Each sac had been filled with a suspension of streptococci from a 16-hour broth culture, suspended in dialyzed peptone, and diluted so that each milliliter contained approximately 48,000 microörganisms. Treatment of the first animal was begun four hours after implantation of the sacs, and of the second animal, eight hours after this operative procedure. Therapy consisted, as usual, of the subcutaneous injection of 20 ml. of a 2 per cent solution of sulfanilamide every four hours. In all, eighteen doses were given to each rabbit over a period of seventy-two hours. In the case of the first rabbit, the temperature began to fall in the middle of the experiment, and the hind legs became paralyzed. For this reason, the rabbit was given a 10-ml. infusion of glucose in saline and the eleventh dose was cut in half. The temperature promptly returned to normal, the paralysis almost disappeared, and the schedule of

treatment was resumed. The concentration of sulfanilamide in the blood was determined at various times during the course of the experiments, in order to ascertain the level, one hour after an injection and again immediately before the subsequent injection. The figures are given in chart 1. Approximately 0.1-ml. samples of sac contents were obtained at intervals of eight hours for the first two days, and exactly 0.05-ml. portions were diluted as high as 1:1,000,000 by the method of serial dilu-

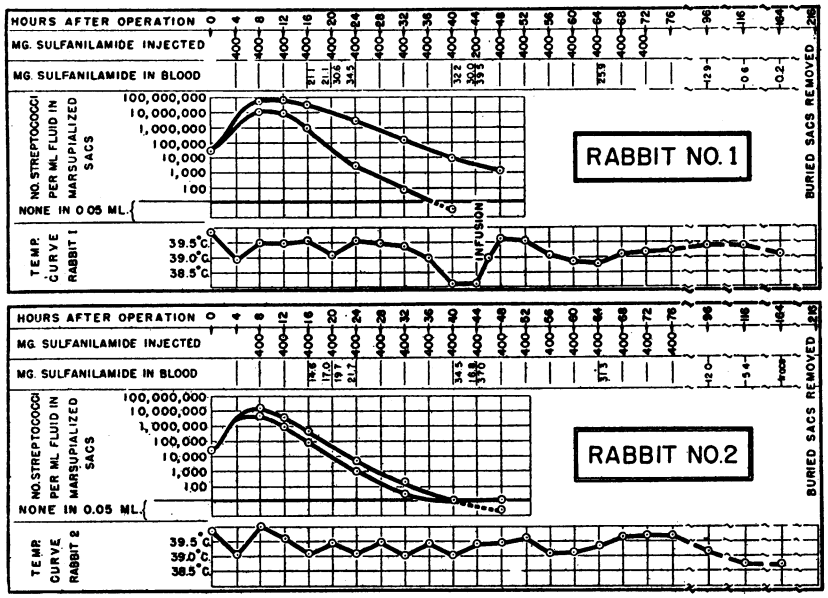


CHART 1. EXPERIMENT 7

tion using multiple pipettes, in order to obtain an estimate of the concentration of streptococci in the sacs at the time of each aspiration. Maximum numbers of streptococci in fluid obtained from the marsupialized sacs in each rabbit were as follows: rabbit 1, sac 1, 65,000,000 per milliliter; sac 2, 27,200,000; rabbit 2, sac 1, 11,200,000; sac 2, 9,150,000. At the end of the 48-hour period very few viable streptococci remained. (See chart 1 and lower part of plate 1.) Nevertheless, therapy was continued for another twenty-four hours in the hope of obtain-

ing complete sterilization of the fluid in the buried sacs. This result was obtained. In order to be certain of this fact, a lapse of six days was allowed between the time of discontinuance of therapy and the termination of the experiment, so that ample opportunity was afforded for complete dialysis out of the sacs of the remaining sulfanilamide, and the proliferation of any viable microörganisms whose growth might have been suppressed by the presence of the drug. Five-tenths milliliter amounts of the contents of each of the buried sacs were injected into individual mice. All remained alive and well. Poured plates were made from the fluid, which was then centrifugalized; the sedi-

TABLE 3

Experiment 7. Contents of buried sacs at end of experiment, 6 days after last injection of sulfanilamide

	MICE INOCULATED WITH 0.5 ML. OF SAC CONTENTS	CULTURE OF CENTRIFUGED SEDIMENT	RESIDUAL SULFANILAMIDE	PRECIPITATION REACTIONS USING SUPERNATANT FLUID	
				Heller	Anti-rabbit-serum hen serum
Rabbit 1:					
Buried sac 1.....	Survived	No growth	0	Negative	Negative
Buried sac 2.....	Survived	No growth	0	Negative	Negative
Rabbit 2:					
Buried sac 1*....	Survived	No growth	0	Positive	Positive
Buried sac 2.....	Survived	No growth	0	Negative	Negative

* Defective.

ment was plated on blood agar; the plates were incubated aerobically for five days at 37°C. No growth occurred. Films prepared from the sediments revealed the presence of a few short chains morphologically characteristic of hemolytic streptococci, but the viability of these microörganisms had evidently been lost. The contents of one of the sacs contained much precipitable protein and many white cells, an imperfection in the sac membrane thus being indicated. The results obtained in this particular instance are, therefore, without significance. The supernatant fluid from the remaining three buried sacs failed to give a positive reaction to either precipitation test at the end of one hour.

There was no residual sulfanilamide detectable in any of the sacs. The results are summarized in table 3. Of interest in this final experiment was the fact that all of the sulfanilamide in the blood of these two rabbits appeared to be in the free form. In view of the observations of Marshall, Cutting, and Emerson (1937) to the effect that rabbits conjugate a considerable percentage of the injected drug, another rabbit was inoculated with a portion of the same solution of sulfanilamide that had been used in this experiment. Blood samples taken from this rabbit indicated that 42 per cent of the total drug was in the conjugated form. These considerations suggest an idiosyncrasy on the part of the rabbits used in the final experiment.

SUMMARY AND CONCLUSIONS

Large numbers of hemolytic streptococci contained in collodion sacs in the peritoneal cavities of rabbits were killed under the influence of sulfanilamide injected subcutaneously, in the absence of phagocytes and, frequently, of precipitable protein.

The results tend to support the prevailing opinion that temperature is a factor of highest importance in the action of sulfanilamide, but they also indicate that phagocytosis, while in all probability complementary in its effect, is not essential in the mechanism of action of the drug *in vivo*.

REFERENCES

ASHESHOV, IGOR. 1933 Study on collodion membrane filters. Part II. J. Bact., **25**, 339–357.

BROWN, RACHEL, AND ROBINSON, L. K. 1938 Supplementary note on ultrafiltration in the preparation of pneumococcal polysaccharides. J. Immunol., **34**, 61–62.

HARRIS, A. H. 1939 A collodion sac for use in animal experimentation. J. Bact., **38**, 321–329.

HARRIS, A. H. 1939 Survival of gonococci in collodion sacs in rabbits. J. Bact., **38**, 241.

HARRIS, A. H., AND MILLER, J. K. 1940 Effect of sulfanilamide injected subcutaneously into rabbits upon hemolytic streptococci contained in collodion sacs implanted intraperitoneally. J. Bact., **39**, 48.

LOCKWOOD, J. S. 1938 Studies on the mechanism of the action of sulfanilamide. III. The effect of sulfanilamide in serum and blood on hemolytic streptococci *in vitro*. J. Immunol., **35**, 155–194.

LONG, P. H., AND BLISS, E. A. 1939 Clinical use of sulfanilamide and sulfapyridine and allied compounds. New York, Macmillan, 319 p.

MARSHALL, E. K., JR., CUTTING, W. C., AND EMERSON, KENDALL, JR. 1937 Acetylation of para-aminobenzene sulfonamide in the animal organism. Science, **85**, 202-203.

WHITE, H. J., AND PARKER, J. M. 1938 The bactericidal effect of sulfanilamide upon Beta hemolytic streptococci *in vitro*. J. Bact., **36**, 481-498.

WHITE, H. J. 1939 The relationship between temperature and the streptococcidal activity of sulfanilamide and sulfapyridine *in vitro*. J. Bact., **38**, 549-562.

WOLFE, H. R. 1936 The specificity of precipitins for serum. J. Immunol., **31**, 103-118.

PLATE 1

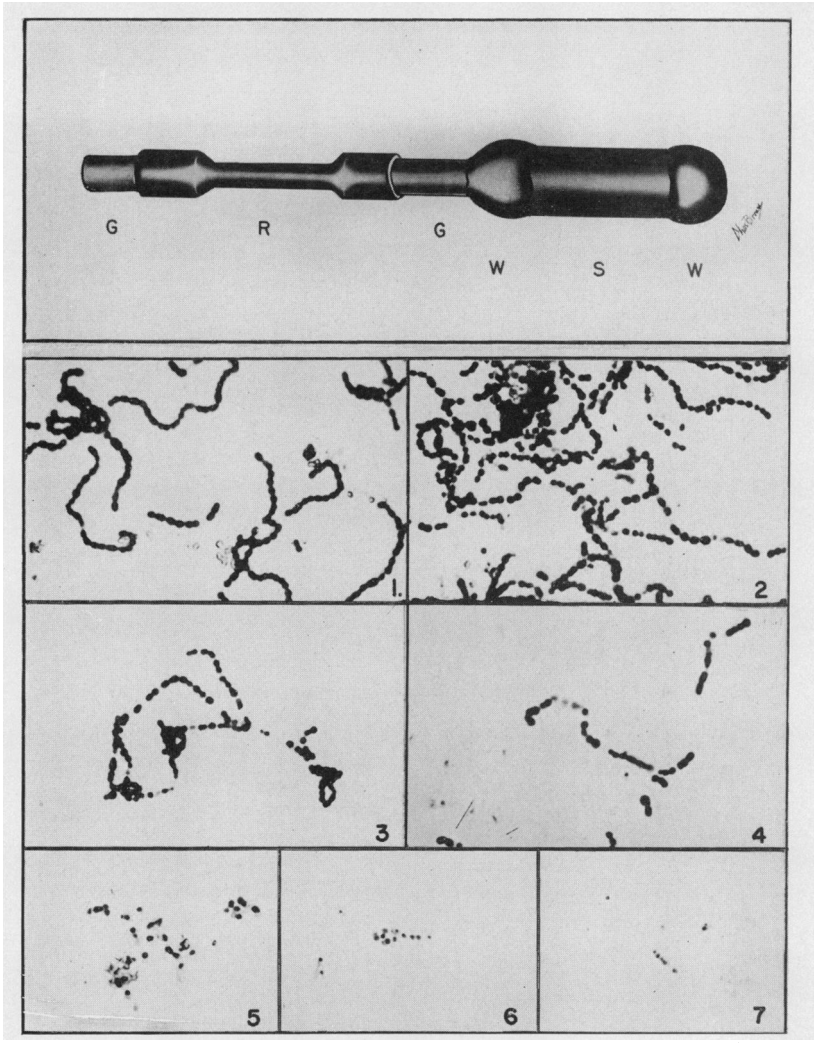
Upper Portion

Drawing of marsupialized sac: *G*, glass tubing; *R*, rubber tubing; *S*, stainless steel gauze supporting collodion membrane; *W*, wax caps.

Lower Portion

Photomicrographs of gram-stained films prepared at intervals from a drop of the contents of marsupialized sac 2, rabbit 2, experiment 7. × 960.

1, 8 hours after operation, and before beginning drug therapy; *2*, 12 hours after operation (4 hours after beginning drug therapy); *3*, 16 hours after operation (8 hours after beginning drug therapy); *4*, 24 hours after operation (16 hours after beginning drug therapy); *5*, 32 hours after operation (24 hours after beginning drug therapy); *6*, 40 hours after operation (32 hours after beginning drug therapy); *7*, 48 hours after operation (40 hours after beginning drug therapy).



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