ON THE MODE OF ACTION OF SULFANILAMIDE IN EXPERIMENTAL STREPTOCOCCUS EMPYEMA*

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PLATE 14

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Little doubt remains as to the chemotherapeutic activity of the substance now conveniently designated sulfanilamide (para-amino-benzene-sulfonamide) and its derivatives, in experimental and naturally occurring infections due to the streptococcus and perhaps other living disease agents. Some form of this drug would seem destined to assume an as yet not fully defined place in the treatment of infections. An increasing appreciation of the therapeutic possibilities of this drug has followed rapidly on the striking experimental results of Domagk in 1935 (1). These results, however, were arrived at empirically, as has been the case with the discovery of most of the chemotherapeutic agents, and further analysis along practical lines has outstripped any definite information as to the precise method by which this substance acts therapeutically in the animal body.

The very fact that this particular drug, although highly effective in the body, has no bactericidal effect on the streptococcus when added to nutrient media in the test tube, a fact that we have ourselves repeatedly verified, at once points to a necessary adjuvant or determinative action on the part of host fluids or host cells. Despite extended investigation, knowledge of the mechanism of host participation in chemotherapeutic action in general, has lagged far behind practical therapeutic results (*cf.* 2). In the particular case that we are considering the possibility of cell intervention in sulfanilamide action has been considered from the very beginning. Domagk (1) noted an increase of mononuclear cells in the peritoneal exudate of

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treated mice, and thought that the reticulo-endothelial system might participate more fundamentally in the drug action. Colebrook and Kenny (3) mentioned a similar possibility without experimental confirmation. Levaditi and Vaisman (4) also noted a macrophage increase but found that blockade by colloidal copper combined with splenectomy in mice did not decrease the chemotherapeutic action. A similar negative effect of splenectomy alone has been described by Gross, Cooper and Peebles (5). With less well defined emphasis, Rosenthal (6) and Long and Bliss (7) have mentioned increased phagocytosis as probably adjuvant to the action of sulfanilamide but this has been specifically denied by Mellon, Gross and Cooper (8).

Our long experience (9) in the study of experimental streptococcus infections in animals has led us increasingly to the conviction of the importance of the slowly mobilizable cells of the macrophage series in natural resistance and in acquired immunity to this microorganism. It was natural then that in analysis of a presumable cell intervention combined with a chemotherapeutic agent, particularly one that is specifically active against the streptococcus, we should turn to an experimental syndrome, streptococcus empyema in the rabbit, which has served as a basis of our study for many years.

EXPERIMENTAL

We have repeatedly described the method we have employed in provoking an extending and rapidly fatal empyema by injecting minimal amounts of a culture of hemolytic streptococcus "H" originally derived from man and still retaining its human characteristics (10) in spite of passage for nearly twenty years through the pleural cavities of some 200 rabbits. This culture in a dosage of not over 10 chains in an 18 hour broth culture (dilution 10^{-7}) seeded directly from the conserved pleural fluid of a fatally infected rabbit usually kills in 4 to 6 days, on direct intrapleural inoculation. The virulence has remained relatively fixed for several years.

Sulfanilamide[1] when given rabbits in relatively large amounts, be-

[1] We have employed for the most part the preparation known as prontylin (para-aminophenylsulfonamide) which the Winthrop Chemical Company has kindly prepared for us in crystalline form without the excipient employed in the tablets designed for oral administration.

ginning a few hours before intrapleural infection with 1000–2000 M.L.D., and continued for at least seven doses during the first 2 days, aborts the otherwise fatal empyema. It requires three daily subcutaneous doses of 20 cc. each of a 2 per cent solution of the sulfanilamide crystals dissolved in boiling water and cooled to body temperature, that is to say, 1.2 gm. of the drug daily, for at least 2 days or a total of almost 3 gm., to effect complete protection against the streptococcus. Smaller total amounts of the drug will at times protect, that is to say, will lead to the complete sterilization of the pleural cavities and blood stream, but assured protection requires the larger dosage indicated. Even larger total amounts of the drug may be given without fatal result although they produce well defined symptoms such as respiratory difficulty, reduction of the red. blood cells and loss of weight. We have, for example, administered a total of 10.8 gm. of sulfanilamide to a 2400 gm. rabbit within 10 days without fatality.

Obviously we are dealing here only with a preventive action induced by sulfanilamide and not with a true curative effect. We have found, to be sure, that a rabbit in which treatment was begun 24 hours after infection survived for 11 days as contrasted with the uniform death of controls in 4 to 6 days. Another rabbit, with treatment beginning 48 hours after infection, died in 5 days, like the control. Our interest at this point lies solely in the study of the mechanism involved under conditions in which the drug is effective, irrespective of any practical therapeutic bearing it may have.

Action of Sulfanilamide as Tested with the Serum of Treated Animals in Vitro

It is generally agreed that sulfanilamide and its derivatives have little if any direct effect on streptococci when added to culture media. On the other hand it has been found that the blood of man and animals that have been treated with the drug may have a distinct inhibitory effect on the microorganism as contrasted with normal blood. As illustrated in our own experiments this bacteriostatic effect is transitory and never results in complete destruction of even a few chains of streptococci.

Experiment 1.—0.1 cc. of a 1–1,000,000 dilution of an 18 hour streptococcus "H" culture (15 chains) was added to 1 cc. of the fresh blood serum of a normal rabbit. The same amount of culture was added to the serum of a rabbit that had been given four 20 cc. doses of 2 per cent sulfanilamide, from 26 to 3 hours before obtaining the blood. 0.1 cc. of these culture mixtures was plated out at intervals on blood agar in successive dilutions and the resultant colonies counted.

TABLE I

Action of Fresh Serum from a Sulfanilamide Treated and a Normal Rabbit on Streptococcus "H"

	Number of chains per cc.			
	5 hrs.	15 hrs.	44 hrs.	92 hrs.
Treated rabbit's serum 1 cc. + 0.1 cc. (10^{-6}) broth culture streptococcus "H" (±15 chains)	4000	12,000,000	11,000,000	60,000,000
Normal rabbit's serum 1 cc. + 0.1 cc. (10^{-6}) broth culture streptococcus "H" (±15 chains)	11,500	500,000,000	370,000,000	90,000,000

It is evident from Table I that although the streptococci are initially (44 hours) inhibited in growth over the control, the number of colonies at the end of 4 days is practically the same.

This contrast in action of drug treated rabbit's serum can be definitely increased if whole defibrinated blood or aseptically produced pleural fluid is employed and if the tubes are continually agitated in a shaking machine. In no instance, however, does the culture become sterile or is the resulting number of colonies at the end of several days remarkably different from controls with normal fluids.

An experiment with serum only has been used for illustration as we wished to contrast this bacteriostatic effect *in vitro* of the fluid of the treated animal free from its cells, with what takes place in the body of a similar animal.

In all instances successful abortion of the rabbit empyema syndrome depends on the repeated injection of the drug and one might well question whether successive doses of sulfanilamide treated serum to a given culture dilution might not result in complete sterilization.

Experiment 2.—0.1 cc. of a 1–1,000,000 dilution of an 18 hour streptococcus "H" broth culture (15 chains) was added to 1 cc. of fresh blood serum of a normal

rabbit. The same amount of culture was added to the serum of a rabbit that had been given four 20 cc. doses of 2 per cent sulfanilamide, from 26 to 3 hours previous to obtaining the blood. At six intervals over a period of 6 days, 0.5 cc. of each mixture was plated out on blood agar in successive dilutions and the colonies counted. Following removal of the serum for plating, 0.5 cc. of fresh corresponding serum, either control or drug-treated, was added to the remaining culture mixtures.

TABLE II

Effect of Successive Additions of Fresh Serum from a Sulfanilamide Treated and a Normal Rabbit on Streptococcus "H"

	Number of chains per cc.					
	5 hrs.	15 hrs.	44 hrs.	92 hrs.	120 hrs.	144 hrs.
Treated rabbit's serum 1 cc. + 0.1 cc. (10^{-6}) broth culture streptococcus "H" (±15 chains)	2600	3,000,000	1,000,000	95,000	1,100,000	65,000,000
Normal rabbit's serum 1 cc. + 0.1 cc. (10^{-6}) broth culture streptococcus "H" (±15 chains)	3700	26,000,000	480,000,000	90,000,000	400,000,000	500,000,000

This experiment shows clearly that although successive additions of drug treated serum result each time in fresh inhibition of the streptococcus growth, and in spite of the fact that the total number of organisms is divided in half before each addition of serum, the culture fails to become sterile. It is clear then that sulfanilamide cannot produce its maximal therapeutic effect simply as a chemical dissolved or transformed in the fluids of the body.

The streptococcus that has been checked in its development in sulfanilamide serum shows distinct degenerative changes that are illustrated in Fig. 2. The chains elongate markedly and the individual cocci are swollen, and metachromatic in the sulfanilamide serum culture, as contrasted with their growth in normal serum (Fig. 1). These distorted cells, however, are still to a degree capsulated. Al-

though the sulfanilamide treated cocci on initial plating may show more "matt" colonies than the "mucoids" that are characteristic of the strain grown in normal serum, on subculture the matt colonies revert at once to mucoid.[2] Much more important is the fact that the culture treated repeatedly with the serum of sulfanilamide treated rabbits has lost none of its virulence.

Thus in two different experiments cultures of streptococcus were prepared by growth in successive additions on the one hand of fresh normal rabbit serum, and on the other, of serum of a rabbit given several doses of sulfanilamide in the manner just described. After determination of the number of viable organisms in each culture mixture, dilutions were made in such a way as to insure approximately the same number of chains of the two different organisms.

A series of rabbits was inoculated with dilutions at 10^{-7}, 10^{-6} and 10^{-5} of the two cultures; that is, 10–13 chains, 100–130 chains and 1000–1300 chains. The two rabbits given the smaller amounts of the two cultures survived, whereas those with the more concentrated dilutions died of typical pleurisy. There is evidently then no essential loss in virulence in a sulfanilamide-serum treated streptococcus.

Action of Sulfanilamide in Rabbit Empyema

Preventive and continued treatment of rabbits with sulfanilamide in sufficient doses prevents the evolution of experimental streptococcus pleurisy as already stated. It remains to follow in more detail the changes that accompany this disappearance of the streptococci in the body of the infected animal.

For the purpose several series of rabbits, both normal and treated, were infected intrapleurally with comparable multiple doses (1000–10,000 times the lethal dose) of streptococcus "H," and killed at intervals of 12, 24, 36, 48, 60 and 72 hours, that is to say, up to the period just before death naturally occurred in the controls. Treated animals were killed at later intervals to compare the findings with those of the controls as the latter died.

The examinations included repeated leucocyte counts of the blood during life in selected cases. At death or on sacrifice of the animal cell counts were made on the exudate or washings from the infected pleural cavity with estimation of

[2] We owe these observations to Mrs. Dorothy W. Miles.

the relative proportions, total number, and the condition of polymorphonuclear and mononuclear cells. Cultures were not only taken from both pleural cavities and the blood stream to indicate the extension of the process, but in the case of the infected pleural cavity the total number of living chains of cocci was estimated by plating. Histological studies were made in particular of both the visceral and the parietal pleura and in several instances of other organs, particularly of the liver, spleen and bone marrow. We present herewith a mere summary of the results obtained in these examinations.

Changes in the Blood.—In control non-treated animals the cultures from the blood have been found positive for streptococcus in every instance from 12 hours onward. There is a drop in the total leucocyte count from 20 hours onward of from one-third to one-half the original number, and from 24 hours onward distinct degenerative changes were noted in the polymorphonuclear cells. From 48 hours onward the mononuclear cells increase to from two to five times the original number.

In sulfanilamide treated animals the blood cultures are uniformly sterile. There is an increase in the total leucocyte count during the early period of infection which reaches from one and one-half to twice the original number of cells up to a period of, roughly, 40 hours. No degenerative changes in the polymorphonuclears were noted. From 48 hours onward, as in the controls, the mononuclear cells increase from two to three times the original number. This relative and actual increase in mononuclears persists long after the infected pleural cavity has become sterile and the total leucocyte count has returned to normal.

The Infected Pleural Cavity.—In the control untreated animal streptococci injected in the pleural cavity increase rapidly. In an animal killed in 12 hours, 6500 times as many organisms were found as had been injected; another at 24 hours gave the same number of multiples; in 48 hours estimates in two animals gave 8000 and 20,000 times the original number. Cultures from the left (uninoculated) cavity were positive in all instances from 12 hours onward. The amount of fluid in the cavity is found markedly increased from 24 hours onward to the time of death, the range being from 2 or 4 cc. in 24 hours to 15 or 20 cc. at the end of 6 days. Throughout the series the cells that compose this exudate are predominantly polymorphonuclear, the

mononuclear clasmatocytes, so long as differentiation can be made, giving relative percentages of from 3 per cent to 16 per cent (average of 8). From 48 hours onward the polymorphonuclears are degenerated so as not to be recognizable except by contrast with the mononuclear cells which may still stain fairly well for at least 2 days.

In the sulfanilamide treated animals the pleural cavity reacts quite differently. In contrast to the immediate increase of cocci in the control to 6500 times in 12 hours, in the treated animal a single animal showed an increase of only 10 times. In the 24 hour animals all three that were examined were positive in the right cavity but in two of these that were plated out the increase was $\times 3$ in one and a decrease in the other to such an extent that 0.1 cc. of the 1 cc. of broth used to wash the clean cavity gave no colonies on a plate, although a slightly larger amount in broth finally became positive. A single 36 hour animal gave exactly the same result as this last animal. Of three animals killed at 48 hours two were completely sterile and one gave a reduction to 150 colonies for the entire cavity from the 37,000 originally introduced. All subsequent cultures from the right cavity in fully treated animals that were observed from 72 hours onward remained completely sterile. With the exception of one animal killed at 12 hours and another at 48 hours, the left (uninoculated) cavities never yielded positive streptococcus cultures.

The amount of exudate in the inoculated pleural cavities of the treated animals was in sharp contrast to that observed in the control animals. In only one instance (48 hours) was any measurable amount of fluid present; estimates in the others, both of the streptococcus and of the exudate, were made by introduction, agitation and removal of a small amount of sterile bouillon. The relative cell counts showed a comparatively small number of polymorphonuclear cells throughout this series since the clasmatocytes ranged from 6 to 73 per cent (average 33 per cent) during the sterilizing period of 48 hours. This resembles the findings noted by Gay and Morrison (11) who found that rabbits protected by preparation with broth showed essentially normal pleuras after infection, although in this case the sterilization (11)[3] was accomplished within 24 hours. A further

[3] Gay and Morrison (11), table 6.

contrast between the exudates in treated and untreated animals, apart from the volume and the relative proportions of cells, lies in the superior condition of both types of cells in the treated series. This is evident even in the exudates of animals insufficiently treated with sulfanilamide that die after the control death period of streptococcus invasion. In the control animals the more labile polymorphonuclears begin to show distinctive degenerative changes as early as 24 hours although the lower percentage of clasmatocytes remain relatively intact for 24 hours longer. Throughout the treated series both types of cells remain relatively normal in appearance. In the treated series phagocytosis by mononuclears is evident and red-staining (dead) chains are notable (Wright stain). This is apparent in spite of the fact that the restricted number of cocci present in the treated animals makes the organisms difficult to find.

Histological Basis of the Resistance Induced by Sulfanilamide

Our previous studies (12) on enhanced local resistance of the pleura to virulent streptococcus, particularly as produced by various inert substances such as broth, aleuronat and gum arabic, have clearly demonstrated that it is due to accumulations of mononuclear cells in adjacent tissues. These results have been amply confirmed by numerous observers. It is natural then that we should have sought for histological changes, whether local or general, in rabbits protected from streptococcus empyema by means of sulfanilamide. Our previous studies had led us to the conviction that the mononuclear cells accumulated in the subserous layers of the pleura were largely local in origin, that is to say, developed from the clasmatocytes (Ranvier), or tissue macrophages, of the connective tissue. We have found no evidence of passage of these cells through the general circulation from more remote areas of the reticulo-endothelial system. We confess to no profound study of such tissues as the spleen, liver and bone marrow in search of a possible remote origin of such cells in our previous work. In connection with this work on sulfanilamide we have, however, examined not only the circulating leucocytes in infected and normal animals, with and without drug treatment, but also the organs that have been mentioned. We have found no histological evidence from this study that sulfanilamide acts through

a general stimulation of the reticulo-endothelial system, a conclusion in agreement with the blockade and splenectomy studies of others that have been mentioned in the introduction.

There remains, however, the possibility that sulfanilamide itself stimulates the local accumulation of mononuclear cells in the pleural wall whenever an irritant is injected into the pleural cavity. To test this possibility we have undertaken two experiments.

Experiment 3.—Twelve adult rabbits were given each 3 cc. of 5 per cent aleuronat plus 3 per cent starch in the right pleural cavity. Six of these rabbits were given subcutaneous injections of sulfanilamide (20 cc., 2 per cent) three times daily beginning 4 hours previous to the aleuronat injections. The other six received none. The drug injections were continued until 3 hours before the animals were sacrificed. Three animals of each series were killed within 24 hours after the aleuronat injections; two each at 48 hours, and one each at 72 hours. The exudates and sections from lung and parietal pleura were studied from the viewpoint of total numbers and relative properties of cells.

The histological picture on comparing these two series was indistinguishable, except as regards individual variations. In 24 hours in both sulfanilamide and control series the exudate and the subserous accumulations of cells are predominantly polymorphonuclear; in 48 hours the cells are mixed, and in the 72 hour animals mononuclear cells predominate. If anything, the mononuclear cells in the non-treated aleuronat series exceeded on an average those in the sulfanilamide aleuronat series. The conclusion is that this drug does not accelerate or increase mononuclear cells in the pleural wall when a sterile irritant is used.

The further possibility exists, however, that the peculiar relationship between mononuclears, sulfanilamide and streptococcus might give different results if streptococci were the irritant employed. To test this possibility we undertook a further experiment.

Experiment 4.—In this double experiment, a small series, three each, of sulfanilamide treated and untreated rabbits were given 500 million formalin killed and washed streptococci in the pleural cavity and representatives of each series sacrificed at 13, 24 and 48 hours. In a second group of treated and untreated animals, two injections of killed streptococci were given at intervals and the animals in pairs were sacrificed 6 hours after the last streptococcus injection.

Exudates and sections from the parietal pleura from the two series gave individual differences that seemed notable but that were not consistent as contrasting between the two series. We are unconvinced that in this particular experiment sulfanilamide treatment showed distinctive stimulating effect for mononuclear cells even in the presence of streptococcus protein.

The failure to demonstrate any distinctive mobilizing power for mononuclear cells on the part of sulfanilamide when the pleural cavity is irritated either by an indifferent substance or by streptococcus protein, renders the consistent histological findings in infected animals cured by sulfanilamide more striking.

We have studied with particular completeness and in sufficient numbers the pleural tissues (visceral and parietal) throughout the critical period (12 to 72 hours) during which cure is established in the sulfanilamide treated animals, and by the end of which time deaths began to occur in the controls.

At the 12 hour stage the only change noted in the tissues in representatives of both series was congestion with slight hemorrhage of the vessels of the serosa but without distinctive cell accumulations.

At 24 hours a difference in the two series begins to appear. Although polymorphonuclear cells predominate in both they are present in larger numbers in the sulfanilamide animals and are found deeper down among the muscle bundles of the parietal wall. A considerable number of mononuclear cells was also noted in one of the treated animals. The sharp histological differentiation begins, however, at 36 hours and continues onward with increasing emphasis.

In the control animals the polymorphonuclears predominate throughout the life of the animal and become increasingly degenerated until death. Necrosis in the muscle bundles begins to appear. Although mononuclear cells appear in small numbers and are at first relatively intact they too become degenerate in appearance by 72 hours (Fig. 3).

Among the treated animals a few mononuclears are evident as early as 24 hours and from 36 hours onward they are the increasingly predominant cell. They are found massed deep down among the muscle bundles and particularly along the septa as well as in the sub-

serous layer of connective tissue which has thickened to accommodate them. The subserous layer of the visceral pleura (Fig. 4), which is very thin normally, thickens notably and nodules of mononuclear cells accumulate around the adjacent alveoli. These cells (septal) are at times in active mitosis. These mononuclear cell accumulations are not so striking at the exact period (48 hours) when complete sterilization of the cavity has just occurred and they are still interspersed with polymorphonuclear cells. They become more notable from 4 days onward (Figs. 5, 6 and 7) and most marked of all in those cases where death was merely delayed until the 9th or 11th day through inadequate treatment.

DISCUSSION AND CONCLUSIONS

Sulfanilamide prevents the evolution of an invariably fatal streptococcus empyema in rabbits when it is given repeatedly and in sufficient doses subcutaneously. Complete sterilization of the inoculated cavity occurs on approximately the 2nd day. The serum, defibrinated blood and artificial pleural exudate of similarly treated animals inhibits the growth of the same streptococcus in the test tube but even repeated doses of such treated blood serum fail to sterilize the culture. The coccal chains grown in such drugged serum are elongated and present pleomorphic and metachromatic organisms and may give rise to colonies that are at first less predominantly mucoid in appearance. Such organisms have, however, lost little if any of their virulence.

Cooperation on the part of locally derived clasmatocytes is apparently required in complete sterilization of the animal body. This conclusion is reached not only by a process of exclusion from comparison with the test tube results, but through the direct histological demonstration of a precocious and increasing mobilization of clasmatocytes in the parietal and visceral pleura of treated animals.

In other words, sulfanilamide apparently produces a bacteriostasis sufficiently marked to protect the accumulated leucocytes and to allow the natural defense macrophages to accumulate. There is direct evidence that the drug does not in itself stimulate the mobilization of the macrophages. There is no evidence that the cell reaction

which finally accounts for disposal of the organisms is other than local.

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EXPLANATION OF PLATE 14

FIG. 1. Streptococcus "H," grown for 92 hours in three successive additions of fresh normal rabbit serum. × 1700.

FIG. 2. Streptococcus "H," grown for 92 hours in three successive additions of fresh serum from a sulfanilamide treated rabbit. × 1700.

FIG. 3. Visceral pleura and lung of untreated control rabbit 16-75. Killed 48 hours after intrapleural infection with streptococcus. Zenker (without acetic acid) fixation. Eosin and methylene blue. × 96.

Extensive polymorphonuclear exudate on surface of the lung. Serosa merely suggested by wavy line. Infiltration of subserous layer and adjacent alveoli by polymorphonuclears.

FIG. 4. Visceral pleura and lung of sulfanilamide treated rabbit 7-06. Killed 48 hours after infection with streptococcus. Fixation, staining and magnification (× 96) as in Fig. 3. No exudate. Serosa intact. A slight but almost entirely mononuclear infiltration of cells in subserous layer.

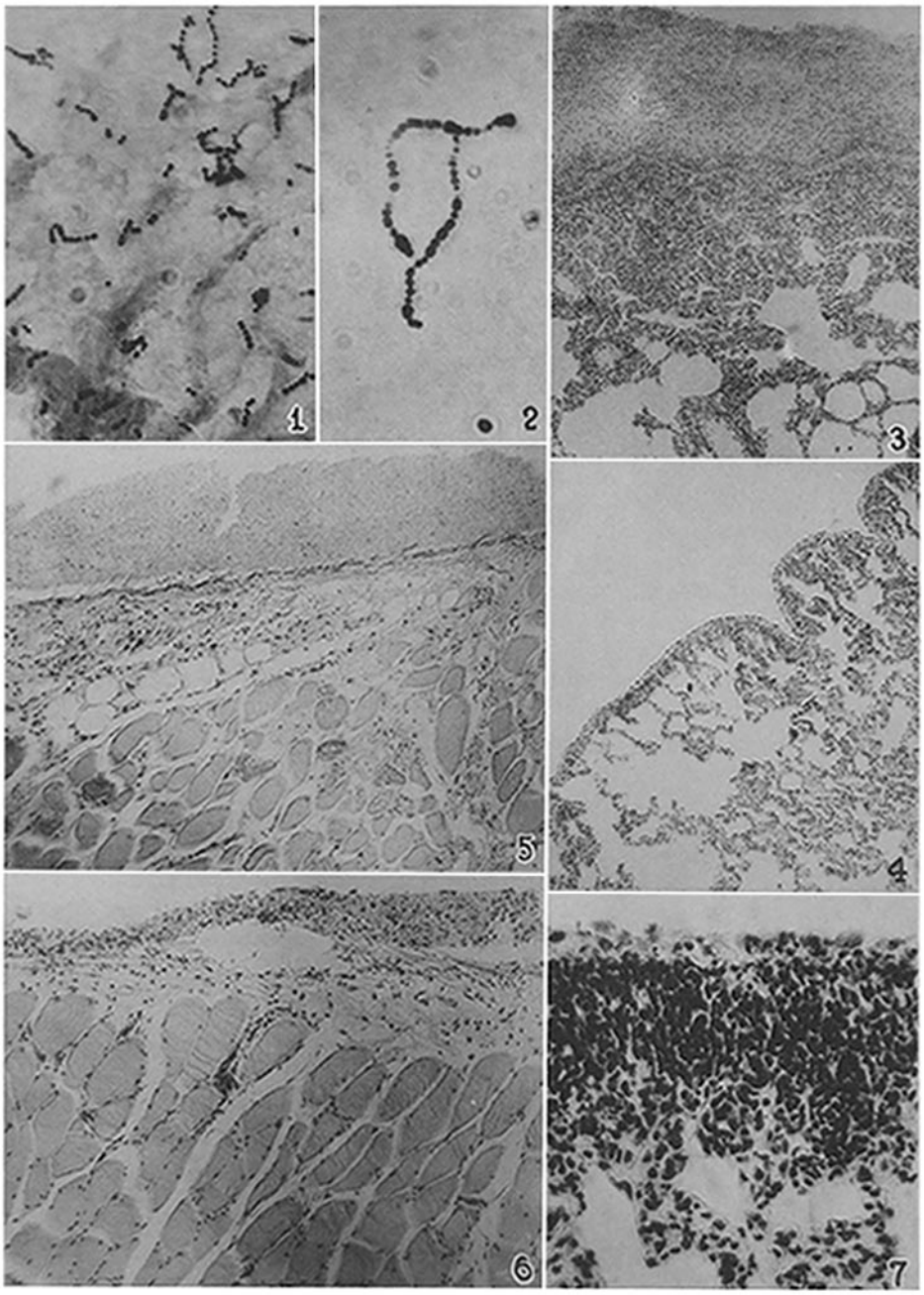
FIG. 5. Parietal pleura of control rabbit 16-67. Death 90 hours after infection with streptococcus. × 144.

A thick necrotic exudate on surface with no intact cells left. Few cells in subserosa, mostly polymorphonuclears, and for the most part with pyknotic nuclei. Adjacent muscle bundles necrotic.

FIG. 6. Parietal pleura of rabbit 16-64 treated with sulfanilamide and killed 90 hours after infection. Cultures all sterile. No exudate. Serosa somewhat thickened. Moderate infiltration of mononuclear cells in widened subserous layer. Nests of mononuclear cells between normal muscle bundles. × 144.

FIG. 7. Visceral pleura of same rabbit (No. 16-64) as in Fig. 6. × 412.

No exudate. Serosa normal. Dense masses of mononuclear cells in subserous layer apparently arising from alveolar walls. Terminal bronchi (not shown in illustration) are in places filled with mononuclear cells.



(Gay and Clark: Sulfanilamide in streptococcus empyema)