STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

II. A Method for Demonstrating the Growth-Inhibitory and Bactericidal Action of Normal Serum-Leucocyte Mixtures.

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The details of the phenomenon of immunity to pneumococci, natural and acquired, active and passive, are not yet entirely clear. The exact rôle which the blood plays in this phenomenon is not established. Certain facts, however, are known.

It has been generally held that the blood serum of naturally immune animals, such as the pigeon, does not confer immunity or protective properties on the susceptible animals. Bull and McKee (1), however, in 1921 reported experiments which indicate that the serum of normal chickens is capable of protecting mice and guinea pigs against infection with moderate sized doses of pneumococci. The blood serum of the animal which has been highly immunized artificially, however, is capable of conferring high grades of passive immunity on a susceptible animal. In this case the properties upon which resistance depends apparently reside largely in the blood serum.

Many attempts have been made to discover the mode of action of the serum of immunized animals in preventing or curing infection. It has been well established that the serum from the artificially immunized animal has certain properties not possessed by the serum of normal animals. It possesses the power of agglutinating pneumococci and of favoring phagocytosis; in other words, it has agglutinating and bacteriotropic powers. Its power of inducing agglutination has been thought by Bull (2) to play an important rôle in its protective action. Other observers think that the increased bacterio-

tropic action of the serum is of chief importance and that phagocytosis is the most important mechanism of which the immune animal makes use in guarding against infection. On the other hand, it has been impossible to demonstrate that immune serum or whole fresh blood of resistant animals possesses any bactericidal power or even power to inhibit the growth of pneumococci (Barber (3)).

In 1912, however, Wright (4) reported that when pneumococci are mixed with blood taken directly from the vessels of certain human individuals, large numbers of pneumococci are killed. According to him this phenomenon does not occur when the bacteria are mixed with whipped blood, or blood which is prevented from clotting by precipitating the calcium. He called this power the phagocytobactericidal power, thereby indicating that the leucocytes are an essential factor in this destructive action. The technique employed in this investigation is not accurately described but apparently the capillary pipette method was employed. It is obvious that in drawing any conclusions in regard to destructive action of serum or blood the effects of agglutination in causing an apparent decrease in numbers of bacteria must be taken into consideration.

In 1918 Heist and Solis-Cohen (5), employing a modified capillary pipette method, reported that when whole pigeon's blood before it coagulates is mixed with small numbers of pneumococci the bacteria fail to multiply. The failure to multiply was determined by blowing the clots out of the capillary tubes on to a slide and examining the stained specimen under the microscope. If instead of pigeon blood, rabbit or mouse blood was employed the bacteria grew with great vigor. This difference between the blood of immune and susceptible species as regards action on pneumococci was not seen if the blood was defibrinated before the bacteria were added. The writers considered the possibility that the difference between uncoagulated and defibrinated blood might depend on the fact that in the former the leucocytes and bacteria may remain distributed in the meshes of the clot and so phagocytosis be favored, but they think this fact would not explain the difference between the action of pigeon and rabbit blood.

Heist and Solis-Cohen (6) extended their studies with this method and found that the ability of a given strain to grow in rabbit blood was directly proportional to the virulence of the strain. Furthermore, they found that failure to grow in the blood of artificially immunized animals bore a direct relation to the degree of immunity possessed by the animals from which the blood was obtained. In blood taken from animals undergoing immunization to pneumococci the destructive action could be demonstrated before agglutinins appeared in the blood. It would seem, therefore, that the apparent failure to grow could not be related to agglutination.

Bull and Bartual (7), using a somewhat similar technique, showed that while the whole blood of immune animals is not so pneumococcidal as had been claimed,

nevertheless, when the blood of naturally immune animals is employed the latent period is prolonged as contrasted with the latent period when the blood of susceptible animals is used. Their observations showed, however, that artificial immunization did not cause the blood to acquire the property of prolonging the latent period of growth. They concluded that the retardation of multiplication which may occur depends upon the opsonization of the pneumococci and phagocytosis. The experiments did not indicate that the bacteria were actually killed since none of the cultures became sterile.

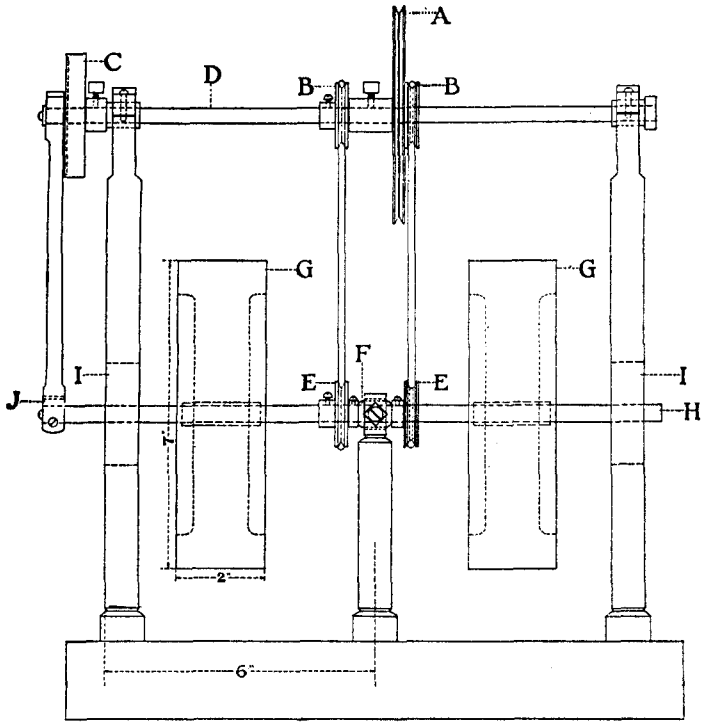
The experiments we have mentioned, therefore, do not certainly indicate whether the apparent retardation of growth in whole blood is simply the result of agglutination and phagocytosis or whether a new and previously unknown factor in immunity is operative.

We have considered the matter of sufficient importance to be investigated further and have attempted to modify the technique employed so as to imitate more closely the actual conditions present in the living animal. In the animal, fluid currents operate to bring the leucocytes and implanted microorganisms into intimate contact. In the capillary pipette, or test-tube, this constant mixing process is absent. Not impossibly, growth inhibition and death only occur if all the organisms come into contact with actively functioning leucocytes and are phagocyted by them. There is a strong probability that in any mixture of whole coagulated blood and pneumococci some of the organisms will escape phagocytosis, and hence, even though growth retardation occurs, the culture ultimately survives. Moreover, in the animal body the blood is not clotted and therefore the experiments carried out with whole blood are conducted under highly artificial conditions. In experiments with whole blood the possibility always exists that the differences observed may depend upon mechanical factors related to differences in the character of the clot formed in the blood of different animals. We have, therefore, not employed whole blood, but have used a serum-leucocyte mixture and have kept the mixture in constant motion.

Method.

A method was devised for growth inhibition tests with the pneumococcus, whereby a constant thorough mixing of leucocytes and organisms was obtained during incubation. Mixtures of serum and

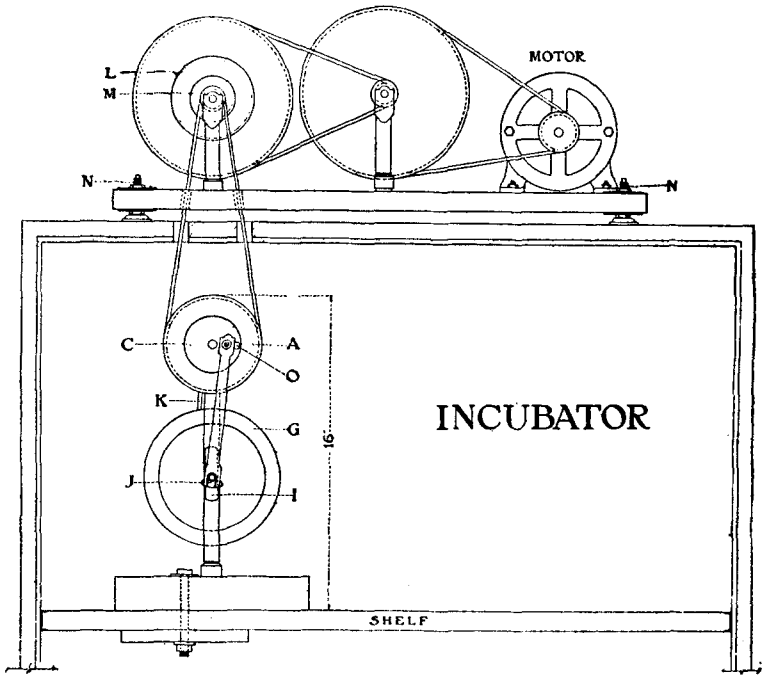
washed leucocytes in small glass tubes were seeded with varying numbers of pneumococci. The tubes were then sealed with paraffined corks, and attached to an agitating apparatus, placed in the incubator.[1]



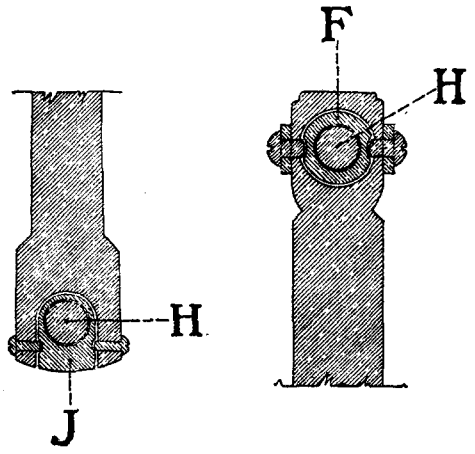
TEXT-FIG. 1. Diagram, drawn to scale, showing apparatus used for agitation. *A*, drive wheel (from motor); *B* and *E*, wheels driving belts from shaft *D* to shaft *H*; *C*, adjustable eccentric; *F*, central pivot-bearing; *G*, wooden wheels to which tubes are attached; *I*, slot in which shaft, *H*, oscillates.

The small drive wheels *B* and *E* are pictured the same size. This is incorrect. *B* wheels are 1 inch in diameter while *E* wheels are $1\frac{1}{4}$ inches in diameter.

[1] Rosenau (Rosenau, E. C., *J. Infect. Dis.*, 1906, iii, 683) describes a method whereby tubes or capillary pipettes containing serum, cells, and pneumococci were shaken in a machine in the incubator. He obtained more constant findings on bactericidal action than when no agitation was employed, but noted a reduction of the number of organisms, not the death of all. The tubes were not sealed; no description is given of the apparatus, and control tests were not provided.



TEXT-FIG. 2. Diagram, drawn to scale, showing apparatus mounted in incubator. *J*, pivot-bearing connecting eccentric with shaft *H; K*, drive belt from *BB* to *EE* (Fig. 1); *L* and *M*, pulley wheels for speed changes; *N*, screw adjustment for tightening belt from pulley system to drive wheel, *A*. The incubator shelf is an ordinary perforated wire shelf through which a bolt passes fixing the base of the apparatus. Purchase for the bolt is obtained with a block of wood.



TEXT-FIG. 3. Drawing showing in detail central pivot-bearing, *F*, and eccentric shaft bearing, *J* (see Text-figs. 1 and 2).

Apparatus.—The apparatus pictured in Text-figs. 1, 2, and 3 and Fig. 1 consists of two solid wooden wheels, equidistant from a central bearing, *F*, on an axle made to oscillate in a vertical plane by means of an adjustable eccentric. The apparatus is driven by a small motor, 1/12 h.p., the speed being reduced to the desired rate by means of a series of pulleys. The speed reduction may also be made by means of a worm-drive direct from the motor to the drive wheel (*A*) of the apparatus. The wheels and eccentric are mounted on a block and placed inside the incubator, with the motor and pulleys on top or above it. The small drive wheels, *EE*, have a diameter one-fifth larger than the wheels, *BB*. From this results a difference in the speed of oscillation and rotation. Two driving belts, *B*, *E*, are employed, because otherwise the small oscillation of the shaft at *E* produces sufficient slackness of the belt at the height of the upward movement to cause slipping. One or the other of the two belts, placed as in the figure, is always tight, thereby insuring constant motion. A flat leather belt, with a piece of tape run through a series of slits cut in the leather (Fig. 1) serves as the holder for the tubes. The means by which the loaded belt is held on the wheel is shown in the illustration. The tape passes under a small metal rod and is then wound on a shaft mounted half an inch above the surface of the wheel and perforated to hold the end of the tape. A rachet wheel with a small detachable handle keeps it in place. It is a simple matter to carry different numbers of tubes in the belt, since the slack of the tape can be taken up.

When the apparatus is in operation, the motion given to the tubes —rotation plus oscillation at a different rate of speed—serves to mix their contents thoroughly and keeps inner surface of the tubes moist, an important matter, because drying would permit some of the organisms to escape phagocytosis. Rotation and oscillation are carried on slowly, so as to reduce to a minimum mechanical injury to the leucocytes.

Constituents of the Test.

Preparation of Organisms.—The growth of the pneumococci, preparation of the standard solution, and method of making the dilutions have been described in the preceding publication (8). Gelatin-Locke's solution, prepared and adjusted to pH 8.0 with $NaHCO_3$, was the suspension fluid.

Strain of Pneumococcus Used.—The organism employed in all the following experiments except 10 and 11, was of Type I, originally isolated from a case of lobar pneumonia and kept in blood broth for several years. Just prior to the experiments the culture was passed through four guinea pigs in quick succession and its virulence for rabbits, guinea pigs, and cats tested, with the following results. Rabbits, 0.00001 cc. of the standard suspension killed; guinea pigs, 0.001 cc. of a heavy broth killed; cats, 1 to 3 cc. of the standard suspension were required to

kill animals of about 2,000 gm. The Type III pneumococcus of Experiments 10 and 11 was one recently isolated from the peritoneal exudate of a case of pneumococcus peritonitis. 4 cc. of the standard suspension killed a 2,000 gm. cat at the end of 10 days. Care was taken that the culture used in the experiments was always in the active growth phase.

Serum.—The serum was obtained for the most part from normal adult animals, by cardiac aspiration. The blood was placed in centrifuge tubes, allowed to clot at room temperature, separated from the walls of the tube, and centrifuged. The serum was removed immediately and kept overnight in the ice box. Occasionally serum was procured on the day of the test. During the latter part of the work, partly for the sake of economy, serum from the animal supplying the leucocytic exudate was used. It was thought that by this means possible untoward effects from blood incompatibility would be avoided. But no difference in results could be made out. Tests for isoagglutinins and isohemolysins were not performed as routine, but in certain instances where they were carried out no evidence of incompatibility was found.

Leucocytes.—The leucocytes were usually obtained from pleural exudate produced by aleuronat, though occasionally leucocytes separated from the circulating blood were employed, as noted in the protocols. 5 cc. of a mixture of equal parts of aleuronat suspension[2] and broth were injected into each pleural cavity of a cat or a rabbit, the species chiefly used in our work. After 24 hours the chest and abdomen were shaved under ether, 5 per cent carbolic acid applied and blood taken for serum and an erythrocyte suspension. For this latter the blood was mixed with an equal quantity of 1 per cent sodium citrate in 0.9 per cent NaCl solution. The animal was then killed with ether, both pleural cavities opened under sterile precautions and 5 to 6 cc. of salt-citrate solution allowed to run into the pleural cavity, the contents of which were transferred to a 15 cc. graduated centrifuge tube while the exudate left behind was taken up with more salt-citrate. In the distribution of it into centrifuge tubes care was taken that the salt-citrate solution was present in at least an equal volume and well mixed with it. A count of the leucocytes was now made on the tube containing the heaviest exudate. 0.1 cc. of the well distributed suspension was taken up into a 0.1 cc. pipette and added to 0.4 cc. salt-citrate solution in a test-tube 1 × 10 cm. containing a glass bead. The pipette was washed out by drawing up the fluid from the test-tube several times. 0.5 cc. of a 0.5 per cent acetic acid solution was next added and after shaking the tube for 5 minutes the leucocytes were counted in the usual way. The standard quantity used in this work was 0.5 cc. of a suspension containing 10,000 leucocytes per c.mm. To determine the number of serum-leucocyte tubes that the total leucocyte suspension would provide, a simple calculation sufficed.

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[2] The aleuronat suspension is prepared as follows: A solution of 1.5 gm. of starch in 75 cc. of water is brought to a boil and 5 gm. of finely powdered aleuronat are slowly added, with constant stirring.

$$\frac{\text{Leucocyte count of suspension}}{10{,}000} \times 2 \times \text{cc. of suspension} = \text{number of serum-leucocyte tubes.}$$

The leucocyte suspensions were next centrifuged at about 1,000 rotations per minute, for 7 to 8 minutes, following which the cells were washed twice, first in gelatin NaCl 0.9 per cent solution, then in gelatin-Locke's solution. Gelatin-containing solutions were used because of Rous and Turner's (9) finding that a small quantity of gelatin served to protect red blood cells against injury when washed. Although these authors did not find that the phagocytic activity of the leucocytes was diminished after washing in non-gelatin-containing solutions, it is possible that the much more highly specialized function of intracellular digestion may well be disturbed thereby. When the yield of leucocytes was small the contents of two or more tubes were combined during the washing. Following it the suspension was made up to a volume such that 0.1 cc. contained 5,000 leucocytes. This concentrated suspension, however, was not made up until after the addition of red blood cells, to be described shortly.

In order to procure leucocytes from the circulating blood, animals were bled from the heart and 4 to 5 cc. of blood distributed into each of several 15 cc. centrifuge tubes containing 6 to 8 cc. of salt-citrate solution, and well mixed. The tubes were then centrifuged for 10 minutes at low speed, the supernatant fluid removed, the leucocyte layer taken in a capillary pipette and transferred to one 15 cc. graduated centrifuge tube, suspended in gelatin-salt solution, and a count made. The later procedure was the same as with leucocytes from an exudate. The red cells were washed in the same manner. 1 cc. of rabbit's or cat's blood will yield enough leucocytes for only one tube or less. From each cc. of guinea pig's blood, on the other hand, sufficient leucocytes can usually be obtained for two tubes.

Red Blood Cells.—Red cells were used as an indicator of bacterial growth. Under conditions of constant agitation, the growth of pneumococci in the presence of blood regularly changes oxyhemoglobin to methemoglobin; and the lack of the change indicates an absence of growth. Given a suitable amount of red blood cells, 0.04 to 0.05 cc. to each tube, the extent of the color change paralleled very closely the degree of growth of the pneumococci during the first 24 to 30 hours of culture. Thereafter color changes from other causes may occur. To insure an identical amount of red cells in each test, the sediment of them, from which the leucocyte layer had been removed, was added directly to the washed leucocyte sediment, which latter usually contained some erythrocytes. The red cells were added in a sufficient quantity to make up a bulk equal to half the volume of the ultimate leucocyte suspension, which was then brought to its final volume with gelatin-Locke's solution.

When for any reason there was a delay in mixing, the leucocytes were placed on ice, whereas the suspension of organisms was kept at room temperature because cold causes the organisms to go into a state of lag. In the early part of the work no attention was paid to the hydrogen ion concentration of the solutions used for

washing and suspending the leucocytes. They were made up from freshly double distilled water sterilized in hard glass flasks and used soon afterwards. Recent work by Evans (10) has shown that the phagocytic activity of leucocytes is depressed by suspension in acid solutions. In the later experiments, therefore, gelatin-salt and gelatin-Locke's solutions were adjusted to a pH 7.2 to 7.5 by the addition of $NaHCO_3$, as described in the previous paper, though with this difference, that no phosphate was added to the gelatin-salt solution. The salt-citrate was found to have a reaction of about pH 7.8 and was used without further adjustment.

Assembling the Test.

Preparation of Small Tubes.—The test-tubes employed were of moderately thin walled glass, 6 cm. long by 7 mm. inside diameter. They were soaked overnight in cleaning fluid, rinsed thoroughly with tap water, boiled in twice distilled water for ½ hour, and scrubbed with hot water and soap. After a second rinsing they were boiled in twice distilled water again for ½ hour, and dried and plugged with cotton. Instead of dry sterilization, the tubes contained in large Petri dishes were autoclaved at 15 pounds pressure for 1 hour and the steam quickly released at the end of this time. Complete drying was accomplished by placing them in a drying oven at 60°C. for 1 hour. Dry sterilization was avoided because not infrequently a hemolytic oil from the cotton is deposited on the walls of the tube when the temperature rises over 140°C., while at times even lower temperatures may cause this to occur.

Preparation of Corks.—Snugly fitting corks with a smooth lower face were chosen for sealing the tubes. They were boiled in twice distilled water for 1 hour, dried, and sterilized in glass containers as with the small tubes, and like them placed in the oven to dry.

Mixing.—The test ingredients were mixed in the following quantity and order.

Serum,	0.2 cc. or 0.3 cc.	+	Leucocyte suspension (containing red blood cells), 0.1 cc.[3]	+	Suspension of organisms,	0.1 cc.

In handling the organisms the highest dilution was added first to all the tubes of that dilution. The same pipette was used throughout and the last 0.1 cc. in the pipette was not delivered. After distributing the organisms, the open end of the tube was flamed thoroughly while held nearly horizontal and was sealed with a cork dipped in hot paraffin. The tube was then rotated rapidly, which allowed the small amount of paraffin carried over on the cork to form a ring at the junction of cork and glass. If the ring failed to form, the cork was redipped. Blowing gently on the cork during rotation hastens the solidification of the paraffin.

[3] Where leucocytes or red blood cells were omitted in the control tubes an equivalent volume of gelatin-Locke's solution was added.

The tube should be flamed only briefly before insertion of the cork, since it is undesirable to have the paraffin run down the inside of the tube. As each tube was placed in the belt it was inverted in order to determine whether or not the sealing was effective. At the same time it was agitated gently so as to moisten the entire inner surface. If too much paraffin is carried in with the cork, the blood will not flow back and forth on tilting. In this event it is desirable to transfer the tube's contents to a fresh tube.

After the test was completely assembled, controls on the organism suspension were planted. 0.1 cc. of the sixth dilution (0.0000001 cc. of the standard suspension) was inoculated into each of two tubes containing 4 to 5 cc. of 1 per cent dextrose blood broth, pH 8.0. Horse blood or rabbit blood was used—0.1 cc. of blood in each tube. If the organisms were in the proper growth phase at the time the suspension was made, abundant growth occurred in 12 to 15 hours. Failure to grow indicated that the suspension was not suitable for the test, and the results were therefore discarded.

The rabbit blood agar plates employed were also tested for their suitability as culture medium. One or two controls were always made on the fluid portion of the medium, *i.e.* serum plus gelatin-Locke's solution, to show that these ingredients of the test mixtures did not cause growth inhibition. Repeated virulence tests of the culture used showed that as small quantities as 0.0000001 or 0.00000001 cc. of the standard suspension always killed mice.

In the majority of the early experiments agitation was maintained at about 18 to 20 revolutions and 8 to 10 oscillations per minute,[4] but in some of the later tests at 15 to 20 per minute. (See page 238 for optimum rate of agitation.) The eccentric was so adjusted that the shaft, *H*, oscillated in the slit, *I*, through a distance of approximately 1½ inches, or an arc of 10 to 11°. The incubator was maintained at 36–37°C. A small electric fan placed inside served to keep an even temperature throughout it. The tubes were observed several times during the first hour's agitation, since there is a tendency for small bubbles to collect at the cork end of some, owing to an excess of paraffin. Their presence is obviously undesirable. A gentle tap will dissipate them, but if they recur persistently, the contents of the tube should be transferred to a fresh tube.

Method of Determining Results.—In determining the results of the tests: (*a*) stained films were made from the tubes to determine whether bacteria were present microscopically; (*b*) cultures were made to determine whether any living bacteria were present; (*c*) in certain instances this was controlled by injection of a definite portion of the contents of the tubes into susceptible mice; (*d*) the change in color of the hemoglobin in the red blood cells in the mixture, due to the formation of methemoglobin, was also used as an indicator of growth of the pneumococci present.

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[4] The experiments in the first part of this work were performed with an agitator, other than that described, in which the ratio of rotation to oscillation rate could be varied at will. This was found to have no advantage over a fixed ratio.

EXPERIMENTAL.

Resistant Animals.

Experiment 1.—(Table I.) Cat serum and leucocytes from the circulating blood were used, procured on the day of the test. Continuous agitation was kept up for 72 hours; revolutions 18 to 20 and oscillations 8 to 10 per minute. The blood pigment changes noted during the first 24 hours. After 72 hours the tubes were opened and stained films carefully examined for pneumococci. The contents of each tube were then transferred to 4 to 5 cc. of 1 per cent dextrose blood broth pH 7.9 to 8.0. These cultures were examined at intervals of 6 to 12 hours during a period of 48 hours incubation.

TABLE I.

Cat serum 0.2 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Color change. At hrs.			Pneumococci in stained film at 72 hrs.	Growth in culture at 72 hrs.
	12	18	24		
cc.					
0.01	++++			+	
0.001	0	±	++++	+	
0.0001	0	0	0	0	0
0.00001	0	0	0	0	0
0.000001	0	0	0	0	0
Control without leucocytes.*					
0.000001		++++		+	

Color changes indicating formation of methemoglobin are indicated as follows:

± = first appreciable change—a little darker red.
\+ = well marked change—definitely darker.
++ = moderately deep red.
+++ = very deep purplish red.
++++ = black.

* 0.05 cc. washed red blood cells freed from leucocytes and suspended in Locke's solution were added.

It is seen in Table I that color changes indicating bacterial growth occurred in Tubes 1 and 2 within 12 hours and 18 hours, respectively, but that with quantities of pneumococci representing less than 0.001 cc. of the standard suspension none took place. No organisms were found in these tubes microscopically at the end of 72 hours, and cultures of the tube's contents showed no growth.

In order to exclude the possibility that lack of growth in dextrose blood broth at the end of 72 hours might be due not to actual death of the pneumococci but to certain involutional changes brought about by the long inhibition of growth in the serum-leucocyte mixture, the tubes in Experiment 2 were cultured at the end of 24 hours.

TABLE II.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.			Culture media used.
	Color change.	Pneumococci in stained film.	Growth in culture.	
cc.				
0.01	++	+	+	Dextrose blood broth, 5 cc.
0.001	+	+	+	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	+	+	+	Rabbit blood agar plates.
0.001	0	0	0	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	++	+	+	Dextrose blood broth, 10 cc.
0.001	0	0	+	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
Control with serum only.				
0.000001		+	+	

Experiment 2.—(Table II.) Cat serum and leucocytes used; revolutions 17 to 18 and oscillations 8 to 9 per minute; agitation continued for 24 hours. At the end of this time color changes were noted and the contents of the tubes examined microscopically and transplanted immediately into 1 per cent dextrose blood[5] broth pH 7.9 to 8.0 and also plated in rabbit blood agar pH 7.3, as indicated in the protocol. Plates were made with agar at a temperature of 40–41°C. A

[5] The 10 cc. tubes received 0.2 cc. horse blood each.

control plate made with 0.000001 cc. of the standard suspension showed good growth. The culture tubes and plates were observed at frequent intervals for 68 hours.

The results of this experiment (Table II) are identical with those of Experiment 1. The media used for culturing the tubes at the end of 24 hours was that used for testing the organism suspension. 0.0000001 cc. of the standard suspension seeded into 10 cc. dextrose blood broth gave an abundant growth in 16 hours, indicating that the medium was excellent for the growth of pneumococci. Part of the tubes was transferred into 10 cc. of the test medium to further dilute any inhibitory substance acting in the 5 cc. amounts. Cultures were also made with rabbit blood agar plates, since the possibility existed that a few surviving organisms might grow in this medium and fail to grow in broth. But as in the case of the tubes cultured in broth, all that showed no growth at the end of 24 hours were found to be sterile on plating.

In order to be certain that we were providing a sufficiently sensitive growth medium for testing the viability of the pneumococci, the contents of the tubes in the next experiment were injected into field mice, which had been found very susceptible to the organism used. 0.0000001 cc. injected into mice weighing 18 to 20 gm. killed regularly in 24 to 36 hours.

Experiment 3.—(Table III.) Cat serum and leucocytes used. Media controls were made immediately following the assembling of the test but there was a delay of some 2 hours before the tubes were started at constant agitation. Revolutions 11 to 12 and oscillations 9 to 10 per minute for 14 hours, then agitation slowed down to revolutions, 4 to 5 and oscillations, 4 per minute for the remainder of the 24 hours. At 24 hours color changes were noted, the contents of the tubes were examined microscopically and transplanted into 1 per cent dextrose blood broth, pH 8.0, rabbit blood agar, pH 7.8, and small field mice as indicated in the table. Those tubes showing marked color changes were not cultured. The mice were observed for 12 days.

The results of this experiment show that pneumococci the growth of which had been inhibited in the cat serum-leucocyte mixture cannot be recovered even by injection into such a sensitive culture medium as the mouse. In a repetition of part of this experiment, it was found that 10,000 times the number of pneumococci sufficient

to kill a mouse, failed to kill after a 24 hour sojourn in the cat serum-leucocyte mixture. The experiment also indicates that dextrose blood broth is at least as sensitive an indicator of the viability of the pneumococcus as is the mouse.

TABLE III.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.			Culture media used.
	Color change.	Pneumococci in stained film.	Growth in culture.	
cc.				
0.01	++++	+		Field mice, weight 20 gm.
0.001	0	+	D. 24–36°.	
0.0001	0	+	" 41°.	
0.00001	0	0	Survived.	
0.000001	0	0	"	
0.01	++++	+		Dextrose blood broth.
0.001	+	+	+	
0.0001	0	+	+	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	++++	+		Rabbit blood agar plates.
0.001	+	+	+	
0.0001	0	0	+	
0.00001	0	0	0	
0.000001	0	0	0	
Controls.	Media.			
0.0000001	Serum.	+		
"	Dextrose blood broth.	+ (14°).		
"	Blood agar plate.	+ 80 colonies.		
0.000001	Mouse.	D. 24–36°.		
0.0000001	"	" 24–36°.		

The question arises as to whether or not the findings constitute conclusive proof that the pneumococci were destroyed by the serum-leucocyte mixture. The sole test for the viability of microorganisms in such small numbers lies in their ability to grow. The failure of

the pneumococci of these experiments to show growth in even the most favorable media may be interpreted as signifying death of the organisms.

TABLE IV.

Dog serum 0.3 cc. + dog leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.		
	Color change.	Pneumococci in stained film.	Growth in culture.
cc.			
0.01	++++	+	
0.001	++	+	
0.0001	0	0	0
0.00001	0	0	0
0.000001	0	0	0
Control with serum only.			
0.000001		+	

TABLE V.

Rabbit serum 0.3 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.		
	Color change.	Pneumococci in stained film.	Growth in culture.
cc.			
0.001	+++	+	+
0.0001	++	+	+
0.00001	++	+	+
0.000001	+	+	+
0.0000001	+	+	+
Control with serum only.			
0.0000001		+	

In order to determine whether the blood of other animals resistant to pneumococcus infection possesses pneumococcidal properties, tests were carried out with the serum and leucocytes of the dog.

Experiment 4.—(Table IV.) Dog serum used and leucocytes obtained from the circulating blood; rates of oscillation, 8 to 9 and rotation, 17 to 18 per minute, but agitation continued for only 6 hours; tubes left undisturbed until the end of 24 hours, when they were examined microscopically and cultured in 1 per cent dextrose blood broth pH 7.9, in which 0.0000001 cc. standard suspension grew abundantly in 16 hours.

The pneumococci failed to grow in mixtures of dog serum and leucocytes in quantities smaller than 0.001 cc. of the standard suspension, or about 1 million organisms; that is to say, there was approximately the same degree of growth inhibition as in the case of the cat.

Susceptible Animals.

Experiment 5.—(Table V.) Rabbit serum and leucocytes used; revolutions, 17 to 18 and oscillations, 8 to 9 per minute; agitation continuous for 24 hours, at the end of which time the tubes were examined microscopically.

It is seen that rabbit serum and leucocytes fail to cause any growth inhibition of even such a small number of pneumococci as 0.0000001 cc. The same suspension of organisms was used in this experiment as in Experiment 2, and the tests were run simultaneously under identical conditions.

In order to determine whether a larger number of leucocytes in the rabbit serum-leucocyte mixture would be more effective in causing growth inhibition, double the quantity of them was used in the next experiment. Also a duplicate set of tubes was put up without red cells, so as to permit possibly a more intimate contact between leucocytes and organisms.

Experiment 6.—(Table VI.) Rabbit serum and leucocytes; suspension of leucocytes twice as concentrated as in previous experiments; revolutions, 18 to 20 and oscillations, 8 to 9 per minute; agitation continuous for 24 hours, at the end of which time color changes noted and stained films made from the tubes.

Increasing the number of leucocytes in the rabbit serum-leucocyte mixture failed to produce any growth inhibition of pneumococci.

Experiment 7.—(Table VII.) Guinea pig serum and leucocytes from the circulating blood were compared with rabbit serum and leucocytes. The same suspension of organisms was used in both, and the tests were carried out simultaneously

under identical conditions. Oscillations, 8 to 10 and revolutions, 30 to 40 per minute; agitation continuous for 48 hours; color changes noted at frequent intervals for 48 hours; and at the end of 70 hours incubation the tubes were opened and the contents examined microscopically.

While growth of the pneumococcus in the guinea pig serum-leucocyte mixture was found to be much slower than in the rabbit elements, all the tubes ultimately showed growth. The growth retarding effect may lie in the guinea pig serum alone, since the rabbit serum control grew out much earlier than did the guinea pig serum control.

TABLE VI.

Rabbit serum 0.3 cc. + rabbit leucocyte suspension (double concentration with and without red blood cells) 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Red blood cells.	At 24 hrs.	
		Color change.	Pneumococci in stained film.
cc.	*cc.*		
0.001	0.05	+++	+
0.0001	"	+++	+
0.00001	"	+++	+
0.000001	"	+++	+
0.0000001	"	0	+
5 dilutions as above.	0	*	+
Control with serum only. 0.0000001			+

* No color change on account of lack of red blood cells.

In order to exclude the possibility that failure of the rabbit and guinea pig serum-leucocyte mixtures to cause growth inhibition of the pneumococcus might be due to injury to the leucocytes resulting from prolonged agitation, an experiment was carried out in which the tubes were agitated for only 6 hours.

Experiment 8.—(Table VIII.) Growth inhibiting effect of serum and leucocytes of rabbit, guinea pig, and dog compared. The leucocytes were kept on ice for 24 hours before use. They were suspended in their homologous serum, except the dog leucocytes, which were by mistake suspended in Locke's solution. Oscillations, 8 to 10 and revolutions, 16 to 20 per minute; agitation for 6 hours;

TABLE VII.

Normal serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Media.	Amount of standard suspension.	Color change. At hrs. 14	16	18	20	24	27	42	48	Pneumococci in stained film at 70 hrs.
	cc.									
Guinea pig serum and leucocytes.	0.01	++	++++							+
	0.001		+	++	+++	++++				+
	0.0001			±	±	++	++++			+
	0.00001						±	+++	++++	+
	0.000001							+++	++++	+
	0.0000001							++	++++	+
Rabbit serum and leucocytes.	0.001	++++								+
	0.0001	++	++++							+
	0.00001		++	+++	++++					+
	0.000001			+	+++	++++				+
	0.0000001			±	+++	++++				+
	Controls without leucocytes.*									
Guinea pig serum.	0.0000001						+	++++		+
Rabbit serum.	"			+	++++					+

* 0.05 cc. washed red blood cells suspended in equal quantity of Locke's solution added to control tubes.

then tubes left undisturbed for 24 hours, at the end of which time films were made and cultures of those tubes showing no growth were planted in 1 per cent dextrose blood broth pH 7.9 to 8.0.

TABLE VIII.

Normal serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Media.	Amount of standard suspension.	At 24 hrs.		
		Color change.	Pneumococci in stained film.	Growth in culture.
	cc.			
Rabbit serum and leucocytes.	0.001	+++	+	
	0.0001	++	+	
	0.00001	++	+	
	0.000001	+	+	
	0.0000001	+	+	
Guinea pig serum and leucocytes.	0.01	++	+	
	0.001	+	+	
	0.0001	0	+	
	0.00001	0	+	
	0.000001	0	0	+
	0.0000001	0	+	+
Dog serum and leucocytes.	0.01	++++	+	
	0.001	++	+	
	0.0001	0	0	0
	0.00001	0	0	0
	0.000001	0	0	0
	Controls without leucocytes.			
Rabbit serum.	0.0000001		+	
Guinea pig serum.	"		+	
Dog serum.	"		+	

The results of Experiment 8 show that with 6 hours agitation pneumococci failed to grow in dog serum-leucocytes in quantities less than 0.001 cc., whereas in rabbit and guinea pig serum even 0.0000001 cc. of the standard suspension showed growth at the end of 24 hours. The first part of the experiment was repeated, using freshly obtained rabbit leucocytes. The result was the same.

Further Observations on the Action of Cat Serum and Leucocytes.

The observations were extended to determine more about the individual factors in the results. For the purpose cat serum and leucocytes were employed in the quantities used previously.

1. Rate of Agitation Necessary.—The speeds of oscillation and rotation were varied over fairly wide limits, and those finally adopted as most suitable were oscillations, 15 to 20 and revolutions, 18 to 24 per minute. However, a little slower speed than this still appears to bring about effective contact between pneumococcus and leucocyte while somewhat more rapid agitation does not seem to injure the leucocytes if the agitation is not continued too long. With very slow agitation on the other hand—4 to 6 revolutions and oscillations per minute—the degree of growth inhibition was definitely decreased.

2. Length of Time of Agitation Required. Experiment 9.—(Table IX.) Tubes agitated at rate of revolutions, 24 and oscillations, 20 per minute. After agitation each set of tubes except the last was taken and allowed to stand in the incubator for 24 hours when they were opened, the contents examined microscopically and transplantations made into dextrose blood broth from those showing no growth. The last set of tubes was agitated continuously for 72 hours. The H ion concentration of the broth in all these experiments was pH 7.8 to 8.0.

Table IX shows that in a relatively short time the maximum degree of phagocytosis is effected, as shown by the fact that growth inhibition in tubes agitated for 2 hours was as great as in those agitated for 24 hours. It might be concluded from the result in the 72 hour tubes that continuous agitation for this length of time was advantageous; but it has been found that a certain amount of variation sometimes occurs within the same experiment as shown in Table X, in which tubes agitated for 18 hours showed more inhibition of growth than those agitated for a longer period of time.

3. Rate of Bactericidal Action. Experiment 10.—(Table X.) Type III pneumococcus used; revolutions, 24 and oscillations, 20 per minute; contents of tubes cultured at intervals of time from 3 to 72 hours; agitation continued for 9 hours. Those tubes cultured at periods later than 9 hours were left undisturbed in the incubator until the time of culture. The tubes cultured at intervals earlier than 24 hours were not examined microscopically.

It is realized that the results of Experiment 10 do not constitute conclusive proof that bactericidal action begins to occur as early as 3 hours after placing the pneumococci in contact with the leucocytes.

It would be possible to prove this point only by injuring the leucocytes sufficiently to liberate the ingested organisms. However, the fact that no special precautions were taken in transferring the serum-leucocyte-pneumococci mixtures into the dextrose blood broth, combined with the finding that bactericidal action became progres-

TABLE IX.

Effect of Period of Agitation.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Length of time agitated.	At 24 hrs.		
		Color change.	Pneumococci in stained film.	Growth in culture.
cc.	*hrs.*			
0.001	2, 3, 6, 9, respectively.	+	+	
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
0.001	24	0	0	+
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
			At 72 hrs.	
0.001	72	0*	0	0
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
Controls with serum only.				
0.000001			+	
0.0000001			+	

* Slight hemolysis in all these five tubes.

sively more pronounced as the time went on, reaching a maximum somewhere between 9 and 18 hours, would indicate that lack of growth in the culture signified that even at 3 hours, death of the organism was taking place. A repetition of the experiment gave similar results.

TABLE X.

Rate of Bactericidal Action.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Cultured at	Pneumococci in stained film.	Growth in culture.
cc.	*hrs.*		
0.001	3	−	+
0.0001		−	+
0.00001		−	+
0.000001		−	0
0.0000001		−	0
0.001	6	−	+
0.0001		−	+
0.00001		−	0
0.000001		−	0
0.0000001		−	0
0.001	9	−	+
0.0001		−	+
0.00001		−	0
0.000001		−	0
0.0000001	(Agitation stopped.)	−	0
0.01	18	−	+
0.001		−	0
0.0001		−	0
0.00001		−	0
0.000001		−	0
0.01	24, 48, 72, respectively.	+	
0.001		+	
0.0001		0	0
0.00001		0	0
Controls without leucocytes.			
0.000001		+	
0.0000001		+	

4. Quantity of Serum Required. Experiment 11.—(Table XI.) Type III pneumococcus employed. Revolutions, 24 and oscillations, 20 per minute; agitation continued for 9 hours only and tubes left undisturbed until 36 hours when they were agitated for 1 hour in order to bring out color changes. Those

that showed a change in color were then examined microscopically, and at 72 hours stained films were made of all the tubes. The contents of the tubes were not cultured.

It was found that the quantity of serum used in the previous experiments was considerably in excess of that actually needed. 0.05 cc. serum diluted to 0.3 cc. with gelatin-Locke's was just as

TABLE XI.

Varying the Quantity of Serum.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Quantity of serum.	Quantity of gelatin-Locke's.	Pneumococci in stained film at 72 hrs.
cc.	*cc.*	*cc.*	
0.01	0.3	0	+
0.001	0.3	0	+
0.0001	0.3	0	0
0.00001	0.3	0	0
0.001	0.2	0.1	+
0.0001	0.2	0.1	0
0.00001	0.2	0.1	0
0.000001	0.2	0.1	0
0.001	0.1	0.2	+
0.0001*	0.1	0.2	+
0.00001	0.1	0.2	0
0.000001	0.1	0.2	0
0.001	0.05	0.25	+
0.0001	0.05	0.25	0
0.00001	0.05	0.25	0
0.000001	0.05	0.25	0
Controls without leucocytes.	Serum.		
0.000001	0.1	0.2	+
"	0.05	0.25	+

* 0.15 cc. suspension added through error.

effective when employed in the serum-leucocyte mixture, as whole serum. Higher dilutions of serum were not made in Experiment 11 as it was feared that a medium containing under 20 per cent serum might prove a less suitable one for the growth of pneumococci.[6]

[6] The action of leucocytes suspended in inactivated serum will be dealt with in a subsequent communication.

5. Quantity of Leucocytes Required.—The amount of leucocyte suspension employed in these tests was chosen arbitrarily before any experiments on the influence of the leucocyte quantity had been performed. Later it was found that the number of leucocytes employed was often considerably in excess of the necessary one. However, the effectiveness of the leucocytes appeared to vary with the individual animal providing them. In one experiment, a tenth of the customary quantity of leucocytes sufficed to bring about maximum inhibition of growth. In other experiments this small quantity was found insufficient, and at times the employment of a leucocyte suspension containing even one-half the usual number of leucocytes, 5,000 per c.mm., failed to cause as much growth inhibition as occurred in other cases with a suspension containing 10,000 leucocytes per c.mm. Using twice the standard quantity failed to produce any increased inhibition of growth. The question of variations in the potency of leucocytes from different individuals of the same species is being investigated further.

6. Amount of Agitation and Time of Incubation Finally Adopted.—By means of a large number of tests the fact was determined that agitation for 6 hours at a speed of 15 to 20 oscillations, through an arc of 10 to 11°, and 18 to 25 revolutions per minute, brought about adequate contact between organisms and leucocytes.[7] The tubes were allowed to remain undisturbed until the end of 36 hours, when they were agitated for 1 hour in order that color changes might be brought out in those in which growth had occurred. Such tubes were examined because sometimes pneumococci growing very early in the test will have autolyzed by 72 hours. The tubes were agitated again for several hours just before the end of the 72 hour period when color changes were noted and the tubes examined microscopically. Latterly, cultures of the tube's contents have not been made, since cultures of more than 100 tubes showing no growth microscopically at 72 hours were found to be uniformly negative.

It was not until this manuscript was practically completed that the work of Fenn (11) on the phagocytosis of solid particles, came to our attention. The method of agitation employed in this work is

[7] Very occasionally prolonged agitation seemed to result in a certain amount of leucocyte injury as evidenced by a lessened degree of growth inhibition.

similar in many respects to that used by Fenn. He rotated small tubes containing serum, leucocytes, and particles of carbon or quartz, on a drum in the incubator and found that phagocytosis was much increased by agitation. Tilting the tubes so that a bubble passed up and down the tube during rotation caused a further increase in the number of particles phagocyted, and he observed that within certain limits the degree of phagocytosis depended on the rate of agitation. Our work shows a general agreement with this finding, in that a certain speed of agitation was necessary in order to obtain a maximum inhibition of growth.

SUMMARY AND CONCLUSIONS.

Somewhat discordant results which have been reported by others who have investigated the property of the whole blood of resistant animals to cause inhibition of growth or death of pneumococci have led us to investigate this matter and to develop a new technique in which the conditions as they are present in the animal body are more nearly imitated. The observations already made have rendered it probable that phagocytosis plays some rôle in any destructive power for pneumococcus which whole blood possesses. We have, therefore, employed mixtures of serum and leucocytes in our tests, since when blood is coagulated the conditions become highly artificial. Furthermore, in order to imitate more nearly the conditions in the circulating blood the mixtures have been constantly, though gently, agitated. For this purpose a specially devised apparatus has been employed. The mixtures of serum and leucocytes have been inoculated with varying numbers of pneumococci in the active growth phase and after varying intervals of time the tubes containing the mixtures of serum, leucocytes, and bacteria have been opened, examined microscopically, and cultures made.

Employing this technique it has been found that the growth of pneumococci having low virulence for cats is markedly inhibited in mixtures of cat serum and cat leucocytes. It was impossible to recover pneumococci from the tubes showing no apparent growth, either when the contents were transplanted into various kinds of culture media, or when the contents were injected into mice of a variety highly susceptible to pneumococcus infection. 10,000 times

the number of pneumococci sufficient ordinarily to kill a mouse failed to do so after a 24 hour sojourn in the cat serum-leucocyte mixture. Mixtures of dog serum and leucocytes exert a similar action. The serum and leucocytes of animals susceptible to pneumococcus infection (rabbits and guinea pigs,) on the other hand, failed to injure pneumococci even in extremely small quantities.

These results indicate that the blood of resistant animals, at least of the dog and cat, possesses destructive properties for pneumococci, and that this destructive power is not possessed by the blood of certain susceptible animals. The experiments suggest that natural immunity depends chiefly, if not entirely, upon this property. The leucocytes play an active part in this process, but whether the destruction of the pneumococci occurs entirely within the leucocytes or not is not determined. That the serum also plays a part is shown by the fact that when the serum of resistant animals was inactivated before being used in the serum-leucocyte mixture, the growth of even very small numbers of pneumococci was not prevented.

Further experiments with cat serum and leucocytes were carried out to determine the optimum rate and time of agitation, the amount of serum and leucocytes required, and also the period of incubation necessary for the inhibition of growth and death of the pneumococci to occur.

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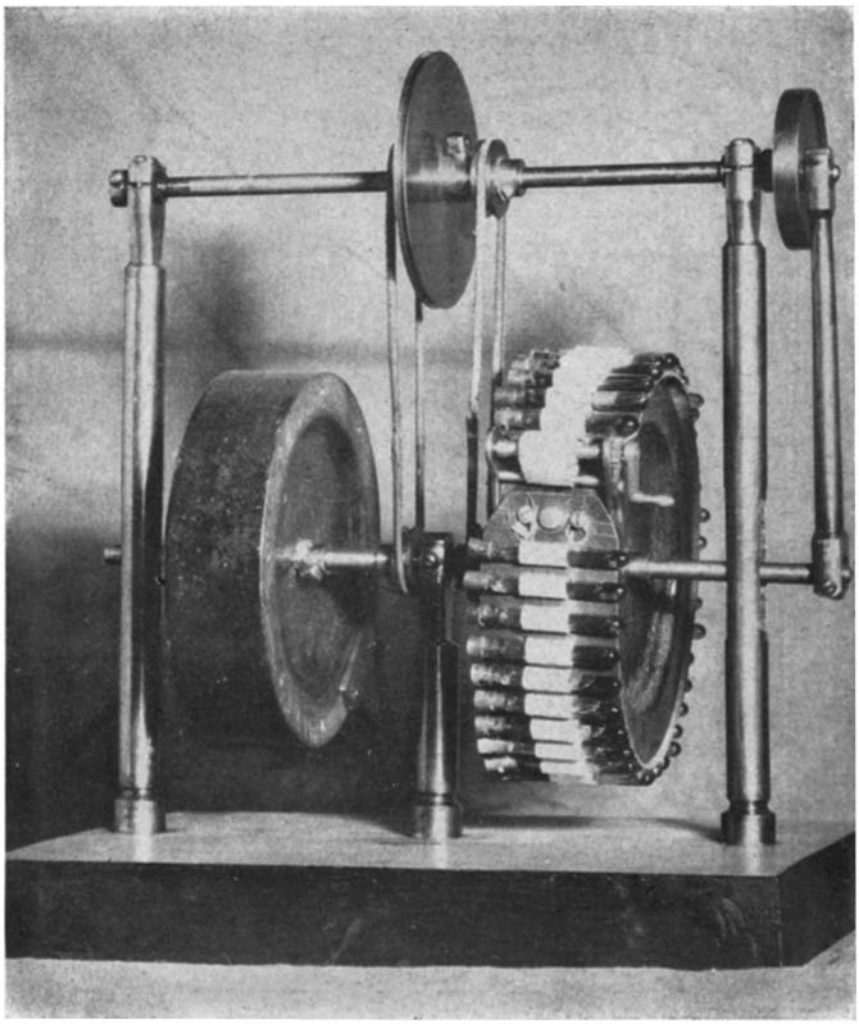
EXPLANATION OF PLATE 20.

FIG. 1. Agitator with belt carrying tubes attached.



(Robertson and Sia: Pneumococcus growth inhibition. II.)