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A STUDY OF THE VIRULENCE OF MENINGOCOCCI FOR MAN AND OF HUMAN SUSCEPTIBILITY TO MENINGOCOCCIC INFECTION[1]

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Epidemic cerebrospinal meningitis presents a number of immunological and epidemiological problems. Chief among these are the rôle of the meningococcus carrier in the dissemination of the infection and the factors that make him a distributor rather than a victim.

Why, in an individual who develops meningitis, do the meningococci invade the deep tissues, and in the carrier confine themselves to the surface of the mucous membrane? Morphologically and culturally the cocci from the two sources are identical; both series are capable of setting up infection in susceptible men and animals; both are agglutinated by polyvalent, antimeningococcic serum.

In searching for an explanation, two phenomena must be considered. One is the *virulence of the meningococci* for human beings; and the other is the *susceptibility of human beings* to the attacks of meningococci. It is important to recognize that these are two separate and distinct phenomena—that the distinction is not one of phrasing, only. We have not to do, as in

[1] Read before the joint meeting of the American Association of Pathologists and Bacteriologists and the American Association of Immunologists, at Cleveland, Ohio, March 25, 1921.

The preliminary work required by this study was made possible through the kindness of Mr. Samuel S. Fels of Philadelphia. The experiments with meningococci were chiefly carried out by the late Dr. George D. Heist at the Base Hospital, Camp Zachary Taylor, Kentucky, in 1919. To Major Herbert Fox, U. S. A., who commanded the Base Hospital laboratory, that author desired to express his gratitude for kindly advice and support.

the case of *resistance* and *susceptibility*, with two sides of the one shield—with a single problem positively or negatively stated—but with two different problems. The one concerns a variable x, relative to a constant y; the other concerns a variable y, relative to a constant x. In final result, increasing either of these factors may, indeed, be equivalent to decreasing the other; but the process—the mechanism—is different. And it is the latter we have to study.

The virulence for lower animals of the microörganisms in question is known to vary within wide limits, and there is no *a priori* reason why their virulence for man should not vary similarly. That individual human beings (and animals) differ greatly in their susceptibility to all bacterial infections is a matter of common knowledge; and the meningococcic infection does not differ essentially from any other.

The object of the present study is to throw light upon each and both of the two phenomena mentioned: (a) meningococcic virulence and (b) the varying susceptibility of human beings to meningococcic infection.

It will be convenient to consider the last-mentioned, first.

THE WHOLE-BLOOD TEST FOR IMMUNITY MAY BE APPLIED TO MEASURE BOTH BACTERIAL VIRULENCE AND ANIMAL SUSCEPTIBILITY

1. Susceptibility.[2] Several years ago the writers described a test that has been applied successfully by others as well as them-

[2] We use the term *susceptibility* in the common acceptation of *lack of resistance*, without reference to any special factor or factors; high susceptibility being low resistance and low susceptibility being high resistance, and so on. Representing complete susceptibility by S and complete resistance by R, the mathematical formula would be $R + S = 0$; hence $R = -S$ and $S = -R$. So too, if virulence be represented by V and non-virulence by A, then $V + A = 0$, and so on. But to get rid of the minus signs we may take x as a variable indicating *resistance*, which at the point of *maximum susceptibility* becomes equal to 0; and y as a variable indicating *virulence* which at the point of *avirulence* becomes equal to 0. If then i represent *immunity* we have the formula $\frac{x}{y} = i$ and the value of i will vary *inversely* as y with a constant x, and *directly* as x with a constant y; while the effect of *multiplying* y by n will be the same as that of *dividing* x by the same factor, and *vice versa*.

selves, to the demonstration, *in vitro*, of the relative susceptibility of animals to various infections—particularly pneumococcic, meningococcic and diphtheric infections. In this test, whole, coagulable blood as it comes from the vessel, is brought in contact with small numbers of bacteria adhering to the inner wall of capillary glass tubes. The bacteria and blood are sealed in the tubes and incubated for twenty-four hours. At the end of that time the contents are blown out and examined microscopically to see whether or not the bacteria have developed.

In a general way, all the writers who have worked with this capillary tube test agree that if the bacteria fail to multiply in twenty-four hours, sufficiently to be seen on microscopical examination, it may be definitely inferred that the animal or man from whom the blood was taken, possesses some degree of immunity against that particular bacterium. In estimating what degree, of course, careful attention must be paid to the essential conditions of the test; particularly as regards the numbers of bacteria seeded in the tubes.

Heist, Solomon Solis-Cohen and Myer Solis-Cohen (1) cultivated pneumococci in the blood of rabbits, mice and pigeons. To quote from the conclusions reached through this study: "If small numbers of pneumococci are seeded, by a suitable method, in pigeon blood before it coagulates, the pneumococci fail to multiply. On the contrary, if pneumococci are seeded in mouse or rabbit blood before it coagulates, the pneumococci grow with great vigor." It will be remembered that the pigeon is immune toward pneumococcic infection, while mouse and rabbit are not.

Bull and Bartual (2) carried the work further. They observed that if incubation of the tubes containing pigeon blood is prolonged sufficiently, growth always occurs, but not until after a period of delay or lag. Lag was absent when mouse or rabbit blood was employed. They found no proof of a true bactericidal action, but considered the phenomena observed to indicate merely a delay or inhibition of growth by the blood of the immune animal.

In a subsequent personal communication, however, Bull states that he has been convinced that the whole fresh blood

of resistant animals may kill a certain number of pneumococci. He gained this impression from the fact that capillary tubes charged with high dilutions of culture and then filled with blood *failed to show growth after any length of incubation period.* He still feels that it is not possible to say definitely whether this result is due to actual killing of the organisms or to an inhibition of multiplication with spontaneous death. The real point, however, is this—and Bull now concedes it—that given a certain relation between the *number* of pneumococci (or other organisms introduced) and the *volume* of blood, the organisms perish in the blood of the immune animal.

Matsunami and Kolmer (3) incubated meningococci in capillary tubes of the whole blood of rabbits and mice. Rabbits are immune from meningococcic infection and mice are fairly susceptible. In rabbit blood little or no growth of meningococci had taken place after twenty-four hours incubation, but in the blood of the mouse, growth was vigorous. Matsunami, in a later communication (4) states that, since the publication of the article referred to, he has found evidence that the action of the immune blood on meningococci is truly bactericidal.

Black, Fowler and Pierce (5), who seeded with undiluted virulent cultures of the typhoid bacillus and of *Bacillus dysenteriae* the blood of rabbits immunized against these organisms and incubated the tubes for different periods of time, found that the organisms were destroyed at the end of five minutes, no bacilli or recognizable fragments being seen in the leucocytes. By means of our method they were able to demonstrate in rabbits the progress of the development of an artificially induced immunity against these organisms; and they conclude, as the result of comparative studies with various methods, that the bactericidal power of blood, thus determined, "is the most dependable criterion of the actual immunity of the animal."

More recently (6) we have studied the behavior of diphtheria bacilli in the whole coagulable blood of certain animals, including the guinea-pig, rat, mouse and rabbit—in each instance using a number of animals of different ages and sizes—and in the defibrinated blood of these species and of the steer. The whole,

coagulable blood and the defibrinated blood of all the animals tested, with the exception of the rat, failed to inhibit the growth of diphtheria bacilli. The whole, coagulable blood of the rat proved bactericidal; its defibrinated blood did not.

2. Virulence. In a second series of experiments, instead of starting with a single virulent strain of pneumococci as they did in the first series, Heist and Solomon Solis-Cohen (7) reversed their angle of approach. They made a normal rabbit their fixed point, and cultivated strains of pneumococci of different degrees of virulence in normal rabbit blood. They found that in such blood virulent pneumococci grew much more readily than did non-virulent ones; from which fact, taken with their measurements and inoculation experiments, they concluded—and this is the conclusion that interests us most at present—that the "mathematical expression" (in terms of the dilution of culture) "of the ability of a strain of pneumococci to grow in the blood of normal rabbits *in vitro* is an expression of the virulence of that strain for rabbits."

Myer Solis-Cohen, Heist and Borow (8) studied the virulence of 88 strains of diphtheria-like bacilli by growing a 1:10 dilution of a suspension of a twenty-four hours serum culture of each strain in guinea-pig's whole, coagulable blood, and noting the shortest time in which growth of the bacteria could be detected. Each strain also was injected into an unprotected guinea-pig and into a control guinea-pig that had received 500 units of antitoxin one hour before the injection of the culture. Fourteen of the cultures failed to grow up within eight hours in guinea-pig's whole coagulable blood, and had no effect when injected in either the unprotected or the control guinea-pigs. These were regarded as *Bacilli hoffmanni.* Seventy-four of the 88 cultures grew up decidedly within eight hours in guinea-pig's whole coagulable blood. Sixty-five of these, which killed only the unprotected guinea-pigs, the controls being saved by the protecting antitoxin, were regarded as virulent Klebs-Löffler bacilli. Three others of the seventy-four cultures that grew up within 8 hours produced in the unprotected guinea-pigs, a transient illness, from which the control pigs

were slightly protected. These were regarded as Klebs-Löffler bacilli that were "slight" or "weak" toxin producers. The remaining 6 of the eight hour cultures killed both the unprotected and the protected guinea-pigs and were regarded as probably identical with the diphtheria bacilli that are usually found at autopsy in the blood and internal organs.

In the case of the pneumococcus, meningococcus and diphtheria bacillus, then, animal *susceptibility* (both as to species and as to individuals of a given species) and bacterial *virulence* (both as to species and individual strains of a given species) may be clearly demonstrated and, to some extent, measured *in vitro* by cultivating the bacteria in whole, coagulable blood.[3]

THE MENINGOCOCCUS PROBLEM

It was thought that similarly, if meningococci from the spinal fluid of cases of meningitis and those from the throats of carriers were grown under like conditions in whole, coagulable human blood, some light might be thrown on their comparative virulence; and that if a strain of meningococci from a severe case of meningitis were grown in the blood of different individuals, some light might be thrown on the comparative susceptibility of men to meningococcic infection.

Before concluding, however, that what is true of animals is likewise true of man, we experimented with bacteria that are known to be practically harmless to men under ordinary conditions, and bacteria known to be commonly harmful to men, to see whether or not the harmful ones would grow more readily in human blood than those that are harmless.

It would have been ideal if throughout this study the different bacteria examined could have been tested against the blood of the same individuals. But this was not practicable. We were obliged to grow each strain in the blood of a different group of persons. The groups were made as large as possible in order that an average might be reached. The tests were made on

[3] We are not here concerned with the explanation of the phenomenon; or with the part played by the factors or process of coagulation; merely with the test and its results.

young men in good health. In addition to the blood test, cultures from the throats of the majority of the groups were studied for meningococci. In two instances carriers were detected, and note of this is made in the tables.

OBSERVATIONS ON COLON BACILLI

The colon bacillus was selected as a type of the ordinarily non-virulent group of organisms. Colon bacilli are sometimes found in diseased human organs, and probably at times initiate morbid processes when introduced into regions other than their normal habitat; but there is no definite colon bacillus malady in the sense that there is a pneumococcus malady or a meningococcus malady.

In this and subsequent experiments our technic as previously described (1) was followed exactly.

A strain of colon bacilli was isolated from feces. The forty-eight hours growth on an agar plate was washed off with sufficient broth to make a dense white emulsion, containing 4 to 6 billion bacilli per cubic centimeter. Four dilutions of this suspension were made in broth, 1:10, 1:100, 1:1000, 1:10,000. The five tubes of a Wright's many-stemmed pipette were filled to a mark; one from the undiluted suspension and one from each dilution. The tips of the tubes were then touched to moist, sterile gauze, and the fluid was permitted to run out, leaving on the inner wall of each tube a film containing bacteria. Blood from a finger prick was immediately filled in up to the mark, and the tubes were sealed and incubated for twenty-four hours. At the end of that time the contents were expelled on to glass slides, stained with dilute carbol-thionin and examined. If great numbers of bacteria were seen in every field a double plus was recorded; if only a few were seen in some fields and in some none at all, a single plus; if only two or three bacteria could be found in the entire preparation, a plus-minus was put down; and if no bacteria at all were seen, a minus.

As a *further control* to each experiment, one set of tubes was loaded with *defibrinated blood* instead of whole, coagulable blood. When growth took place in the defibrinated blood and

not in the whole coagulable blood, we were assured that there was active in the whole, coagulable blood some anti-bacterial factor (or factors) lacking in the defibrinated blood.[4]

TABLE 1

Cultures of Bacillus coli in whole, coagulable human blood

	DILUTIONS OF A HEAVY SUSPENSION OF B. COLI IN BROTH				
	Undiluted	1:10	1:100	1:1000	1:10,000
Man no. 1	—	—	—	—	—
Man no. 2	—	—	—	—	—
Man no. 3	—	—	—	—	—
Man no. 4	—	—	—	—	—
Man no. 5	—	—	—	—	—
Man no. 6	—	—	—	—	—
Man no. 7	—	—	—	—	—
Man no. 8	—	—	—	—	—
Man no. 9	—	—	—	—	—
Man no. 10	—	+	—	—	—
Man no. 11	—	—	—	—	—
Man no. 12	—	—	—	—	—
Man no. 13	—	—	—	—	—
Man no. 14	—	—	—	—	
Controls:					
Man H	—	—	—	—	—
Defibrinated blood	+	+	+	+	+
Dextrose broth	++	++	++	++	++

As will be seen in table 1 almost no growth of colon bacilli had occurred in the whole blood of normal men after twenty-four hours incubation.

OBSERVATIONS ON HEMOLYTIC STREPTOCOCCI

Few microörganisms are responsible for more infections of the human race than are the streptococci. Streptococci are able to invade any and all tissues, setting up severe and ofttimes fatal

[4] Were it not for our previous observations of this phenomenon, it would be astonishing; for *a priori* one would expect defibrinated blood to be the more bactericidal, owing to the release, by the disintegration of leucocytes, of bactericidal endolysins allied to the microcytase of Metchnikoff.

inflammations and intoxications. This may not, indeed, be true of all the Gram-positive, chain-forming microörganisms we are accustomed to call by that name. Some of them may be as harmless as the colon bacillus in its normal domain. But it is certainly true of many of the hemolytic variety. Therefore, if our hypothesis concerning the relation of whole coagulable

TABLE 2

Cultures of Streptococcus hemolyticus from a leg ulcer, in whole, coagulable human blood

	DILUTIONS OF TWENTY-FOUR HOURS GROWTH ON BLOOD AGAR TUBE SUSPENDED IN 2 CC. DEXTROSE BROTH				
	Undiluted	1:10	1:100	1:1000	1:10,000
Man no. 1	++	++	++	++	++
Man no. 2	+	+	+	–	–
Man no. 3	+	–	–	–	+
Man no. 4	++	++	+	–	+
Man no. 5	++	++	++	–	–
Man no. 6	±	±	+	–	–
Man no. 7	+	+	–	+	–
Man no. 8	++	++	++	–	–
Man no. 9	++	++	–	–	–
Man no. 10	–	–	–	–	–
Man no. 11	++	++	++	+	–
Man no. 12	++	++	++	++	–
Man no. 13	++	++	–	++	–
Man no. 14	+	–	++	+	–
Man no. 15	++	++	++	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++
Blood broth	+	+	+	+	+

blood to immunity be correct, a hemolytic streptococcus, fresh from some human infection, should grow in whole coagulable human blood much more readily than the colon bacillus does. To test this a hemolytic streptococcus was isolated from a severe leg ulcer, following a shell wound, and cultured in the blood of a series of men. The result is shown in table 2.

In evaluating this result the difference between the number of streptococci in the fluid from which tube 1 was seeded, and the number of colon bacilli in the fluid from which tube 1 was seeded in the preceding experiment, must likewise be taken into consideration. The undiluted suspension of streptococci contained about one billion bacteria per cubic centimeter, while the undiluted suspension of colon bacilli contained four to six times that number. Yet there were sufficient streptococci present to produce vigorous growth in the blood of all but two of the fifteen men tested, even in the 1:100 dilution; while colon bacilli grew in only one of the entire lot of capillary tubes. This seems to give considerable emphasis to the trustworthiness of the criterion.

But we were not entirely satisfied with this experiment, despite the growth of the streptococci. They did, indeed, grow better than did colon bacilli; yet the whole, coagulable blood of normal men was able to offer considerable resistance to them, as compared with the lack of resistance shown by defibrinated blood. When a virulent pneumococcus is cultivated in the blood of rabbits, it grows just as well in whole blood as in defibrinated blood.

Experiments were made with many bacteria, but in no instance could a parallel be found in man to the pneumococcus phenomenon in the rabbit. The failure is not surprising. The observations were confined to microörganisms such as pneumococci, *Bacillus influenzae* (Pfeiffer), staphylococci, *Bacillus diphtheriae* and others, which ordinarily affect man, and excluded what may be called the artificial or extraordinary infections of man, such as glanders and anthrax. There are not so many infections to which man is universally susceptible, of which the agent is known, and, at the same time, suitable for study by the capillary tube method. Bubonic plague is one, but we were not able to obtain a freshly isolated culture of *Bacillus pestis.*

However, the two experiments recorded are sufficient to prove that what Matsunami and Kolmer, and Bull and Bartual, as well as ourselves, have found to be true of animals, is true of human beings also; namely, that there is a certain correlation

between the ability of bacteria to grow in the whole, coagulable blood of man and their virulence for man. We felt therefore that we could look with favor on the method, as a means of testing the relative virulence of strains of meningococci for human beings.

EXPERIMENTS WITH MENINGOCOCCI

The medium on which the meningococci were grown was beef infusion agar plus 7 per cent horse serum and 1 per cent dextrose. The sterile serum and dextrose solution were added after cooling the agar to 55°C., and the reaction was then adjusted, sterilely, to pH 7.4. The atmosphere in which the meningococci were grown was kept saturated with moisture by closing all the ventilating holes and placing large, shallow pans of water on the floor of the incubator.

The method adopted by the United States Army for the detection of meningococcus carriers was followed, excepting that serum-dextrose-agar replaced blood agar.

It should be stated, specifically, that in this study no coccus with the cultural and morphological characteristics of meningococci was accepted for the work unless it was also completely agglutinated by polyvalent anti-meningococcus serum, 1:100, with clearing of the supernatant fluid after 18 hours at 55°C. Moreover, any trace in the controls of agglutination with normal horse serum, 1:50, or with salt solution, ruled the culture out.

Normal and para serums were used in typing. Distinct agglutination by one serum alone was accepted as sufficient evidence that the strain belonged to that type. Complete sedimentation with clearing was not required. All strains which were not agglutinated by either normal or para serum were called irregular.

After isolation, the meningococci were transplanted at least twice daily and no transplant over twelve hours old was used for the whole, coagulable blood tests. *The use of a young culture of meningococci for tests with whole blood is a most vital point.* Usually but a few transplants intervened between the isolation of the meningococci and their culture in whole, coagulable blood.

The suspensions for the tests were made by washing off the growth of a large agar slant with 2 cc. of warm serum-dextrose broth. All strains grew about equally well on agar so that each suspension contained approximately the same number of cocci. The suspensions were of such density that a wire held between them and the light was barely visible. This corresponded to from four billion to six billion cocci per cubic centimeter. The dilutions were made in warm serum-dextrose broth.

All of the tables covering the meningococcus work have been placed together, with a few clinical notes concerning each case.

Case 1. H. O. White. Aged twenty-one. The attack began with headache and vomiting followed by unconsciousness. The patient was admitted to the hospital March 17 with a temperature of 101°. On March 18 his spinal fluid contained over 5000 leucocytes per cubic millimeter, 95 per cent of them being polynuclears. Gram-negative, intracellular diplococci were seen. They grew well on serum-dextrose agar and proved to be normal meningococci. They were cultured in whole blood, as shown in table 3.

The patient was given serum, intraspinally and intravenously. After the third dose there was a marked improvement in his condition. His recovery was not interrupted by any complication. By April 1 convalescence was well established.

During April, cultures of his naso-pharynx were taken fifteen times. Meningococci were found on five occasions. The positive culture of April 24 was tested with whole, coagulable blood, as shown in table 6.

Case 2. E. B. White. Aged twenty-three. The attack began two days before admission to the hospital, with severe headache and pain in the calves of the legs. A maculo-papular eruption appeared over the entire body. The patient was admitted to the hospital on February 17, with headache and vomiting. His spinal fluid was cloudy and contained over 5000 leucocytes per cubic centimeter, ninety-five per cent of them being polynuclears. No bacteria were seen, but in a similar specimen, drawn the following day, Gram-negative intracellular diplococci were found. They failed to grow on serum-dextrose agar.

From February 17 to April 2 the patient was, alternately, desperately ill and slightly improved. His temperature ranged from 98° to 104°. Serum was given without apparent effect. In all 450 cc. were given

intraspinally and 63 cc. intravenously. At no time could improvement be ascribed to the use of the serum. Meningitis was complicated at different times by pneumonia of one lobe of the right lung, endocarditis, and orchitis.

At times the spinal fluid was heavily clouded and at other times slightly opalescent. Repeated cultures were made from it, but it was not until March 22 that a growth occurred. The organisms proved to be meningococci. They were completely agglutinated by polyvalent serum, but not by either normal or para type sera. They were tested with whole, coagulable blood (table 4).

The patient's temperature reached normal by April 2, and two weeks later he was definitely convalescent.

Frequent cultures were made from his naso-pharynx. A strain of meningococci was isolated on March 1. It was tested with whole, coagulable blood (table 7).

It needs but a glance at these tables to see the marked differences, as to ability to grow in whole, coagulable blood, between meningococci from the *spinal fluid* of the two cases of meningitis and those from the *throats* of the carriers. The strain from the spinal fluid of case 1 (H. O.) (table 3) grew well in whole blood, and the strain from the spinal fluid of case 2 (E. B.) (table 4) grew very well. Among the carrier strains there was some variation. Strains from the throat of carrier M. W. (table 16) and from the throat of carrier G. R. S. (table 18), grew to some extent, but the others scarcely grew at all.

From the reports of others who have used the capillary tube method of testing bacterial virulence, and from our own preliminary experiments with both virulent and non-virulent bacteria, we are justified in concluding that this disparity of growth in whole, coagulable human blood connotes a like disparity in *virulence;* and that while the meningococci from spinal fluids are in general decidedly virulent for man, most of those from the throats of carriers are but slightly so. It is true that some strains from carriers are more virulent than others, and that some strains from spinal fluids are less virulent than others; but the carrier throat-strains are always relatively weak, the spinal-fluid strains always relatively powerful.

TABLE 3

Cultures of normal meningococci, isolated March 18 from the spinal fluid of case 1 (H. O.), in whole, coagulable human blood

	UNDILUTED	1:10	1:100	1:1000	1:10,000
March 19—Dilutions of 8 hours culture:					
Man no. 1	++	++	++	++	++
Man no. 2	++	+	++	−	+
Man no. 3	++	++	++	+	++
Man no. 4	+	+	−	−	−
Man no. 5	+	++	+	−	−
Man no. 6	++	++	+	−	−
Man no. 7	++	++	++	−	++
Man no. 8	++	+	−	−	−
Man no. 9	+	+	−	−	−
Man no. 10	++	+	+	−	−
Man no. 11	++	++	+	−	−
Man no. 12	++	+	+	−	−
Man no. 13	0	−	−	−	−
Man no. 14	++	++	−	−	−
Man no. 15	++	++	−	−	−
Man no. 16	++	++	++	++	++
Man no. 17	++	++	++	++	++
Man no. 18	++	++	−	−	−
Controls:					
Man H	+	+	+	+	+
Defibrinated blood	++	++	++	++	++
March 20—Dilutions of 8 hours culture:					
Man no. 19	++	++	++	++	−
Man no. 20	++	++	++	+	−
Man no. 21*	+	−	−	−	−
Man no. 22	++	++	++	++	−
Controls:					
Man H	++	++	++	++	++
Man K	++	++	++	−	++
Defibrinated blood	++	++	++	++	++

* Proved to be carrier of irregular meningococci.

As to the *relative susceptibility* of different men, we have here observational—to a certain extent experimental—data. The nature of the test probably introduces a fairly large factor of error, so that small differences must be disregarded. But,

TABLE 4

Cultures of irregular meningococci, isolated March 22 from the spinal fluid of case 2 (E. B.), in whole, coagulable human blood

	UNDILUTED	1:10	1:100	1:1000	1:10,000
March 30—Dilutions of 9 hours culture:					
Man no. 1	+	+	−	−	−
Man no. 2	++	+	+	+	−
Man no. 3	++	++	+	+	+
Man no. 4	++	++	++	+	+
Man no. 5	++	++	+	−	−
Man no. 6	+	+	−	−	−
Man no. 7	++	++	++	+	−
Man no. 8	++	++	+	−	−
Man no. 9	++	++	++	−	−
Man no. 10	++	++	++	+	−
Man no. 11	+	+	+	+	+
Controls:					
Man H	+	+	+	+	+
Man K	+	+	++	++	++
Defibrinated blood	++	++	++	++	++
April 3—Dilutions of 12 hours culture:					
Man no. 12	++	++	++	−	−
Man no. 13	++	++	−	−	−
Man no. 14	++	++	++	++	-
Man no. 15	++	++	++	++	++
Man no. 16	++	++	−	−	−
Man no. 17	++	++	++	++	++
Man no. 18	++	++	−	−	−
Man no. 19	++	++	++	++	++
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++
April 4—Dilutions of 5 hours culture:					
Man no. 20*	++	++	++	++	++
Man no. 21	++	++	+	−	++
Man no. 22	++	++	++	++	++
Man no. 23	++	++	++	−	−
Man no. 24	++	++	++	+	−
Controls:					
Man H	0	++	++	++	++
Defibrinated blood	++	++	++	++	++

TABLE 4—*Concluded*

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 7—Dilutions of 8 hours culture:					
Man no. 25	+	+	+	+	+
Man no. 26	++	++	++	++	++
Man no. 27	++	++	+	+	++
Man no. 28	++	++	++	++	++
Man no. 29	++	++	++	++	++
Man no. 30	++	++	+	−	+
Man no. 31	++	++	++	++	++
Man no. 32	++	++	++	++	++
Man no. 33	+	+	−	−	−
Man no. 34	++	++	++	++	++
Man no. 35	+	−	−	+	−
Man no. 36	++	++	++	++	++
Controls:					
Man H	+	−	−	+	−
Defibrinated blood	++	++	++	++	++

* Proved to be carrier of irregular meningococci.

allowing for error, the blood of some men evidently permits the growth of a given strain much more readily than does the blood of other men. We can only conclude that the men whose blood shows little resistance to meningococci are more liable to infection, on exposure, than are those whose blood is more actively bactericidal. The less virulent the strains permitted to grow, the greater the degree of susceptibility.[5]

[5] Since $\frac{(\text{Resistance})\ x}{(\text{Virulence})\ y} = i$, a low value of i should indicate a high value of y but if y is low as well as i then x must have a value approaching or reaching 0. For example, $\frac{x}{y} = \frac{0}{y} = i = 0$; while $\frac{x}{y} = \frac{x}{0} = i = \infty$. If $\frac{nx}{ny} = ni$ then $\frac{nx}{\frac{ny}{2}}$ should equal $2ni$; but if $\frac{qx}{\frac{ny}{2}} = \frac{ni}{4}$ then the value of q (qualifying x) is only $\frac{n}{8}$

TABLE 5

Cultures of irregular meningococci, isolated March 22 from the spinal fluid of case 2 (E. B.), in the whole, coagulable blood of carriers of meningococci

	UNDILUTED	1:10	1:100	1;1000	1:10,000	TYPE OF MENINGOCOCCUS CARRIED
April 10—Dilutions of 8 hours culture:						
Carrier no. 1	++	++	+	–	–	Normal
Carrier no. 2	++	+	–	++	–	Irregular
Carrier no. 3	++	++	++	++	–	Normal
Carrier no. 4	–	++	–	–	–	Irregular
Carrier no. 5	++	++	++	–	–	Normal
Carrier no. 6	++	–	–	–	–	Irregular
Carrier no. 7	++	++	++	++	++	Irregular
Carrier no. 8	++	++	++	++	++	Normal
Carrier no. 9	–	–	–	++	++	Irregular
Carrier no. 10	++	++	++	–	–	Normal
Carrier no. 11	++	++	++	–	–	Normal
Carrier no. 12	+	+	+	–	–	Normal
Carrier no. 13	–	–	–	+	–	Normal
Carrier no. 14	++	++	++	–	–	Irregular
Carrier no. 15	+	+	+	–	–	Normal
Carrier no. 16	++	++	++	–	–	Irregular
Controls:						
Man K	++	++	++	++	++	
Defibrinated blood	++	++	++	++	++	
April 17—Dilutions of 10 hours culture:						
Carrier no. 17	++	++	++	++	++	Para
Carrier no. 18	++	++	+	–	++	Normal
Carrier no. 19	++	++	+	+	++	Normal
Carrier no. 20	++	++	++	+	++	Normal
Carrier no. 21	++	++	++	++	++	Irregular
Carrier no. 22	++	++	++	++	++	Irregular
Carrier no. 23	++	++	+	+	+	Irregular
Carrier no. 24	+	±	+	±	–	Irregular
Carrier no. 25	++	++	++	++	++	Irregular
Controls:						
Man H	++	++	++	++	++	
Man K	++	++	+	++	++	
Defibrinated blood	++	++	++	++	++	

TABLE 6

Cultures of normal meningococci, isolated from the throat of case 1 (H. O.), 38 days after the onset of the infection, in whole, coagulable human blood

	UNDILUTED	1:10	1:100	1:1000	1:10,000
May 9—Dilutions of 8 hours culture:					
Man no. 1	–	–	–	–	–
Man no. 2	+	–	+	–	–
Man no. 3	–	–	–	–	–
Man no. 4	++	–	–	–	–
Man no. 5	–	–	–	–	–
Man no. 6	–	–	–	–	–
Man no. 7	–	–	–	–	–
Man no. 8	+	+	–	–	–
Man no. 9	–	–	–	–	–
Man no. 10	–	–	–	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++

TABLE 7

Cultures of un-typed meningococci, isolated March 1 from the throat of case 2 (E. B.), in whole, coagulable human blood

	UNDILUTED	1:10	1:100	1:1000	1:10,000
March 12—Dilutions of 11 hours culture:					
Man no. 1	±	–	–	–	–
Man no. 2	–	+	–	–	–
Controls:					
Man H	+	+	+	+	+
Defibrinated blood	+	+	+	+	+

TABLE 8

Cultures of normal meningococci, isolated February 18 from the throat of carrier (A. F. W.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined twenty-three days. Throat cultures 10. Positives 2)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
February 21—Dilutions of 9 hours culture:					
Man no. 1	–	–	–	–	–
Man no. 2	–	–	–	–	–
Man no. 3	–	–	–	–	–
Controls:					
Man H	+	–	–	–	–
Defibrinated blood	+	+	+	+	–

TABLE 9

Cultures of un-typed meningococci, isolated February 20 from the throat of carrier (T. R. B.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined twenty-nine days. Throat cultures 13. Positives 3)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
February 21—Dilutions of 9 hours culture:					
Man no. 1	–	–	–	–	–
Man no. 2	–	–	–	–	–
Man no. 3	–	–	–	–	–
Controls:					
Man H	+	–	+	–	–
Defibrinated blood	+	+	+	+	+

TABLE 10

Cultures of normal meningococci, isolated March 14 from the throat of carrier (Y.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined eighty-three days. Throat cultures 30. Positives 15)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
March 17—Dilutions of 12 hours culture:					
Man no. 1	+	+	–	–	–
Man no. 2	++	++	–	–	–
Man no. 3	++	++	+	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	+	+	+	+	+

TABLE 11

Cultures of normal meningococci, isolated March 18 from the throat of carrier (H. T.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined thirty-seven days. Throat cultures 13. Positives 5)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
March 21—Dilutions of 8 hours culture:					
Man no. 1	–	–	–	–	–
Man no. 2	++	++	–	–	–
Man no. 3	++	–	–	–	–
Man no. 4	+	+	–	–	–
Man no. 5	–	–	–	–	–
Man no. 6	–	–	–	–	–
Man no. 7	++	–	–	–	–
Man no. 8	–	–	–	++	–
Man no. 9	–	–	–	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++

TABLE 12

Cultures of normal meningococci, isolated April 5 from the throat of carrier (J. J.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined twenty-five days. Throat cultures 9. Positives 5)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 8—Dilutions of 11 hours culture:					
Man no. 1	+	−	−	−	−
Man no. 2	−	−	−	−	−
Man no. 3	+	−	−	−	−
Man no. 4	−	−	−	−	−
Man no. 5	−	−	−	−	−
Man no. 6	+	−	−	−	−
Man no. 7	−	−	−	−	−
Man no. 8	−	−	−	−	−
Man no. 9	−	−	−	−	−
Man no. 10	−	+	+	+	−
Man no. 11	−	−	−	−	−
Man no. 12	−	−	−	−	−
Man no. 13	−	±	±	−	−
Man no. 14	−	+	−	−	−
Man no. 15	+	+	+	−	−
Controls:					
Man H	+	+	+	+	−
Defibrinated blood	+	+	+	+	+

TABLE 13

Cultures of normal meningococci, isolated April 5 from the throat of carrier (J. S. C.), in whole, coagulable human blood. (Contact of case of normal meningitis. Quarantined thirty-four days. Throat cultures 12. Positives 5)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 8—Dilutions of 11 hours culture:					
Man no. 1	+	−	−	−	−
Man no. 2	+	−	−	−	−
Man no. 3	+	+	−	−	−
Man no. 4	+	+	−	−	−
Man no. 5	−	+	−	−	−
Man no. 6	−	−	−	−	−
Man no. 7	+	−	−	−	−
Man no. 8	+	+	−	−	−
Man no. 9	−	−	−	−	−
Man no. 10	+	+	−	−	−
Man no. 11	+	−	−	−	−
Controls:					
Man H	+	+	+	+	−
Defibrinated blood	+	+	+	+	−

TABLE 14

Cultures of normal meningococci, isolated April 5 from the throat of carrier (P van P.), in whole, coagulable human blood. (Casual from Camp Dix. Quarantined forty-one days. Throat cultures 14. Positives 5)*

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 11—Dilutions of 8 hours culture:					
Man no. 1	−	−	−	−	−
Man no. 2	−	−	−	−	−
Man no. 3	++	++	+	−	−
Man no. 4	++	++	−	−	−
Man no. 5	−	−	−	−	−
Man no. 6	−	+	−	−	−
Man no. 7	+	−	+	−	−
Man no. 8	+	+	−	−	−
Man no. 9	+	+	+	+	+
Man no. 10	−	−	−	−	−
Man no. 11	+	+	−	−	−
Man no. 12	+	+	−	−	−
Man no. 13	−	−	−	−	−
Man no. 14	−	−	−	−	−
Man no. 15	−	−	−	−	−
Man no. 16	+	−	−	−	−
Controls:					
Man K	+	+	+	−	−
Man H	+	+	++	++	++
Defibrinated blood	++	++	++	++	++

* Cultures were taken from the throats of all men transferred to Camp ——— from any camp reporting cases of meningitis, before admission. These men were termed "casuals."

TABLE 15

Cultures of irregular meningococci, isolated April 3 from the throat of carrier (J. P.), in whole, coagulable human blood. (Casual from Camp Dix. Quarantined forty-one days. Throat cultures 14. Positives 5)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 11—Dilutions of 11 hours culture:					
Man no. 1	−	−	−	−	−
Man no. 2	−	−	−	−	−
Man no. 3	++	−	−	−	−
Man no. 4	−	−	−	−	−
Man no. 5	−	−	−	−	−
Man no. 6	−	−	−	−	−
Man no. 7	−	−	−	−	−
Man no. 8	−	−	−	−	−
Man no. 9	−	−	−	−	−
Man no. 10	−	−	−	−	−
Controls:					
Man H	++	++	−	−	−
Defibrinated blood	++	++	++	++	++

TABLE 16

Cultures of normal meningococci, isolated April 10 from the throat of carrier (M. W.), in whole, coagulable human blood. (Casual from Camp Dix. Quarantined seventy-seven days, and cultures still positive. Throat cultures 24. Positives 12)

	UNDI-LUTED	1:10	1:100	1:1000	1:10,000
April 14—Dilutions of 12 hours culture:					
Man no. 1	+	+	−	−	−
Man no. 2	++	++	+	+	+
Man no. 3	+	+	+	−	−
Man no. 4	+	+	−	+	−
Man no. 5	−	−	−	−	−
Man no. 6	−	−	−	−	−
Man no. 7	+	+	−	−	−
Man no. 8	+	−	+	−	−
Man no. 9	−	−	−	−	−
Man no. 10	−	−	−	−	−
Man no. 11	+	+	+	−	−
Man no. 12	+	+	−	+	−
Man no. 13	−	−	−	−	−
Man no. 14	−	−	−	−	−
Man no. 15	+	+	+	−	−
Man no. 16	+	+	−	−	−
Man no. 17	++	++	−	−	−
Man no. 18	++	++	++	+	+
Man no. 19	++	+	−	−	−
Man no. 20	++	++	+	−	−
Man no. 21	−	−	−	−	−
Man no. 22	++	++	−	−	−
Man no. 23	++	++	−	−	−
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++

TABLE 17

Cultures of irregular meningococci, isolated April 12 from the throat of carrier (O. S.), in whole coagulable human blood. (Contact of case of normal meningitis. Quarantined thirty-six days. Throat cultures 13. Positives 4)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 18—Dilutions of 10 hours culture:					
Man no. 1	++	++	–	–	–
Man no. 2	+	–	–	–	–
Man no. 3	++	–	–	–	–
Man no. 4	–	–	–	–	–
Man no. 5	–	–	–	–	–
Man no. 6	–	–	–	–	–
Man no. 7	++	–	–	–	–
Man no. 8	–	–	–	–	–
Man no. 9	–	–	–	–	–
Man no. 10	–	–	–	–	–
Man no. 11	+	+	–	–	–
Man no. 12	++	–	–	–	–
Man no. 13*	+	–	–	–	–
Man no. 14	+	–	–	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++

* Proved to be carrier of irregular meningococci.

TABLE 18

Cultures of irregular meningococci, isolated April 12 from the throat of carrier (C. R. S.), in whole, coagulable human blood. (Casual from Camp Dix. Quarantined thirty-nine days. Throat cultures 15. Positives 7)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 19—Dilutions of 12 hours culture:					
Man no. 1	++	++	++	+	+
Man no. 2	++	++	–	–	–
Man no. 3	++	++	–	–	–
Man no. 4	++	–	–	++	++
Man no. 5	+	+	–	–	–
Man no. 6	++	++	–	–	–
Man no. 7	++	++	++	++	–
Man no. 8	++	++	++	++	–
Man no. 9	++	++	++	–	–
Man no. 10	++	++	++	++	++
Man no. 11	+	+	+	+	+
Man no. 12	+	+	+	+	–
Man no. 13	++	++	–	–	–
man no. 14	++	++	++	–	–
Man no. 15	+	+	–	–	–
Man no. 16	–	–	–	–	–
Man no. 17	++	++	–	–	–
Man no. 18	++	–	++	–	–
Man no. 19	++	++	++	++	++
Man no. 20	++	–	–	–	–
Controls:					
Man H	++	++	++	++	++
Defibrinated blood	++	++	++	++	++

TABLE 19

Cultures of irregular meningococci, isolated April 16 from the throat of carrier (J. G.), in whole, coagulable human blood. (Casual from Camp Jackson. Quarantined thirteen days. Throat cultures 5. Positive 1)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
April 21—Dilutions of 10 hours culture:					
Man no. 1	−	+	−	−	−
Man no. 2	−	−	−	−	−
Man no. 3	−	−	−	−	−
Man no. 4	−	−	−	−	−
Man no. 5	−	−	−	−	−
Man no. 6	−	−	−	−	−
Man no. 7	−	−	−	−	−
Man no. 8	−	−	−	−	−
Man no. 9	−	−	−	−	−
Man no. 10	+	−	−	−	−
Man no. 11	−	−	−	−	−
Controls:					
Man H	++	++	−	−	−
Defibrinated blood	++	++	++	++	−

TABLE 20

Cultures of Para meningococci, isolated April 16 from the throat of Carrier (E. F.), in whole, coagulable human blood. (Casual from Camp Jackson. Quarantined thirteen days. Throat cultures 5. Positive 1)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
May 8—Dilutions of 8 hours culture:					
Man no. 1	−	−	−	−	−
Man no. 2	−	−	−	−	−
Man no. 3	−	−	−	−	−
Man no. 4	−	−	−	−	−
Man no. 5	−	−	−	−	−
Man no. 6	−	−	−	−	−
Man no. 7	−	−	−	−	−
Man no. 8	+	−	−	−	−
Man no. 9	−	−	−	−	−
Man no. 10	−	−	−	−	−
Controls:					
Man H	+	−	−	−	−
Defibrinated blood	+	+	+	+	−

TABLE 21

Cultures of irregular meningococci, isolated April 16 from the throat of carrier (E. F.), in whole, coagulable human blood. (Casual from Camp Jackson. Quarantined twenty-four days. Throat cultures 10. Positive 4)

	UNDILUTED	1:10	1:100	1:1000	1:10,000
May 13—Dilutions of 10 hours culture:					
Man no. 1	–	–	–	–	–
Man no. 2	+	–	–	–	–
Man no. 3	–	–	–	–	–
Man no. 4	–	–	–	–	–
Man no. 5	++	–	–	–	–
Man no. 6	–	–	–	–	–
Man no. 7	–	–	–	–	–
Man no. 8	–	–	–	–	–
Man no. 9	–	–	–	–	–
Man no. 10	–	–	–	–	–
Man no. 11	++	–	–	–	–
Controls:					
Man H	+	+	+	–	–
Defibrinated blood	0	++	++	–	–

SUMMARY OF OBSERVATIONS

In the foregoing observations 172 men were tested with different carrier strains. The whole, coagulable blood of three of these men (control man H, man no. 10 and man no. 19, table 18) had no greater resistance to meningococci than had defibrinated blood. The blood of four others (man no. 9, table 14, man no. 2 and man no. 18, table 16, and man no. 1, table 18) had but little more. If we estimate susceptibility on the basis of these limited figures, it would appear that so far as the resisting power of the blood is concerned, the susceptibility of men in general to meningococcic infection is quite low. We need not be insistent on the exact proportion. Whatever may be shown by studies on a larger number of persons, the percentage is surely small—probably less than five.

Early in this study it was observed that the blood of one of the three men mentioned had very little resistance to meningococci. It was possible to include his blood (lettered "H" in the tables)

as a control, in every experiment. It will be seen that, in all the tables, this man's whole, coagulable blood hindered the growth of meningococci but little, if any, more than did defibrinated blood. This adds much to the value of the experiments when we are considering their reliability as a test of individual susceptibility. The presence of a positive control shows that, given a non-resisting medium, the meningococci can multiply. When they do not so increase, the presence of some bacteriotropic pressure is implied. A second man (lettered "K") was included as a control in several experiments. His blood was usually a little more resistant than that of "H."

Undoubtedly, other factors besides the blood are concerned in protecting an individual from meningococcic infection. The man "H" was constantly exposed to meningococci from cases and carriers but was not apparently infected during the period covered by these observations.[6] At the same time he was exposed to diphtheria bacilli from cases of diphtheria and diphtheria carriers, yet, although he had a strongly positive Schick reaction, he escaped infection from that organism. His whole, coagulable blood was subsequently shown, by means of the capillary tube method, to be highly bactericidal to the diphtheria bacillus, demonstrating that he possessed high bactericidal immunity against the diphtheria bacillus, although lacking antitoxic immunity (6).

UNANSWERED QUERIES

A question at once presents itself: Are meningococci from the spinal-fluid in a case of meningitis and those from the throat of the *same patient* unequally virulent? We were unable to answer this to our satisfaction. From case 1 (H.O.) no throat strain was isolated until the 6th week of the disease, when the patient was convalescing and was quartered in the same ward with carriers. Here his chance of being infected with a strain from another carrier was great. This strain was tested on ten men. There was practically no growth (table 6). From case 2 (E. B.) a throat strain was isolated before a spinal-fluid strain was procured. It was tested on two men only (table 7) with a negative result.

[6] See, however, page 29.

Sufficient evidence has not yet been obtained, upon which to venture an opinion. One may guess that there is disparity, but it remains for future study to prove or disprove. Another interesting question arises: Does the blood of the meningococcus *carrier* have more resistance to meningococci than the blood of normal men? To this we may tentatively answer Yes. Some 25 carriers were tested with the spinal fluid from case 2 (E. B.) (table 5). The blood of the carriers was slightly more resistant than the blood of normal men (table 4). In the earlier stages of the work carrier strains were cultured in the blood of the man from whom the strain came, and, at the same time, in the blood of normal men. Experiments to overcome technical difficulties were then under way but not yet complete, and the results were less sharp than they became later. However, there were distinct indications that the blood of the carrier was decidedly more resistant to his own strain than was the blood of normal men.

DISCUSSION

In this study we have, through experimentation with whole, coagulable blood, arrived at certain conclusions. They are as follow:

1. Meningococci from the spinal fluid are much more virulent for man than are the majority of the strains of meningococci which inhabit the throats of carriers.
2. Some carrier strains are more virulent than others.
3. Men vary greatly as to their susceptibility to meningococcic infection. Some men appear to be quite resistant, others much less so.

Knowledge of the epidemiology of cerebrospinal meningitis is extensive. Let us examine our laboratory results in the light of experience.

Cerebrospinal meningitis is but mildly contagious. Our experiments have shown that the most virulent meningococci are those in the central nervous system of a patient. It would appear to be probable that were these meningococci spread

broadcast, many of those in contact with the patient would become actively infected. But bacteria in the central nervous system have only a limited chance to escape. The nasal mucous membrane is the point of least resistance but it is surely a barrier which requires time to pass. The presence of highly virulent meningococci, deep in the body, does not imply that the malady must be highly contagious.

Among those who have been in contact with a case of meningitis the percentage of carriers is sometimes very high—8 to 12 per cent. But we cannot take it for granted that these individuals have necessarily received their meningococci from the sick man. Both case and carrier may have been infected by a third person. Culturing in whole, coagulable blood indicates that the majority of throat strains are but slightly virulent for the vast majority of normal human beings; that but one among many is susceptible. If this be true, we can build up a picture of what takes place when a carrier is brought in contact with a group of men. The cocci are transferred from man to man. In most cases they do no harm and, after a time, disappear. But if they happen to reach a man with low resistance, they are able to invade the deep tissues and reach the central nervous system—provided, of course, that other unknown factors of immunity are absent.

In the spring of 1919, a Sanitary Train, comprising 119 officers and men, passed through a port of debarkation, all in good health. Twenty-four hours later, while they were on a train bound for the west, one man developed cerebrospinal meningitis. The 119 men were removed from the train and quarantined. Within a few hours cultures were made from their throats and 25 per cent of them were found to be carriers of meningococci. It is hard to imagine how this one man could have received his infection and distributed the meningococci to 29 others during the short incubation period of cerebrospinal meningitis. It is at least more plausible to think that this detachment at some time, probably while still in France, met with a meningococcus carrier. The cocci were slowly passed from man to man, without

doing harm, until they reached the susceptible individual, who promptly came down with meningitis. The 29 carriers were quarantined and their throats cleared promptly under treatment. A number of them are included in this study.

The laboratory observations here recorded do not, then, lead us to conclusions that conflict, at any point, with empirical knowledge concerning cerebro spinal meningitis. They show a little of what takes place in the colloidal realm when man and the meningococcus meet, and may thus assist in attaining to a better understanding of the special factors of immunity and susceptibility toward cerebrospinal meningitis.

The subsequent history of man "H" illustrates the lack of resistance to meningococcal infection that accompanies absence of bactericidal power against the meningococcus. Man "H" was no other than Dr. George D. Heist, the chief author of this paper. His failure to become overtly infected while working among meningitic patients and with meningococci—of great as well as of lesser virulence—may have been due in part to the ordinary precautions a physician takes when working with infective material. With no known contact with patient or carrier,[7] in the absence of any known cases in the city, Dr. Heist in August, 1920, developed epidemic cerebrospinal meningitis, and although the diagnosis was made early and energetic treatment instituted with anti-meningococcic serum obtained from the Rockefeller Institute, injected intravenously, intraspinally and subcutaneously, the patient succumbed—a loss beyond measure to science and to his friends.

The unique interest attaching to the case, suggests the publication of certain particulars.

Dr. Heist was thirty-six years of age. His father had died at the age of twenty-four of typhoid fever, the course of which presented many points of similarity to the fatal illness of the son. Four paternal uncles had died of acute illnesses that were said to have "gone to the head."

[7] Members of his family and his laboratory associates were studied. Cultures from their air-passages gave negative results.

Dr. Heist was always slight and frail. He had been associated with an uncle by marriage, who had recovered from tuberculosis. In 1910 he developed pulmonary tuberculosis and spent a year at Saranac Lake. He had been well since his return and in 1918 passed the physical examination for a commission in the United States Army.

In July, 1919, while working harder than usual, performing autopsies daily, sometimes being so occupied the whole day, he complained of malaise, fatigue, irritability and nervousness, but had no pulmonary symptoms. Physical examination at that time showed tuberculous consolidation of the apex of the right upper lobe and slight infiltration of the apex of the left upper lobe, both probably healed. Under rest and tonic treatment the patient recovered.

In July, 1920, he again had been undergoing unwonted physical exertion, building a summer-house. On the last day of the month, while in a shop, he suddenly became unconscious for half an hour, after which he was dazed, weak and nauseated and had 1° F. elevation of temperature.

He was taken to the Germantown Hospital, where the condition was regarded as heat stroke. He went home that evening, and the following day developed severe headache over the temples and pain over the eyeballs, which persisted, although slightly relieved by medication, which he prescribed for himself.

Two days later the patient vomited, exhibited some stiffness of the neck and was apathetic. The white blood count was 9300, polynuclears 84 per cent, transitionals 0.5 per cent, lymphocytes 15.5 per cent. The temperature had been moderately elevated and the pulse slow. Examination by Dr. John A. Kolmer of 25 cc. of cloudy spinal fluid removed at noon showed 621 cells per cubic centimeter with predominance of polynuclear cells, some red blood cells, increased globulin, reduction of Fehling's solution, and absence of tubercle bacilli. No meningococci were seen on the first examination, but a subsequent examination of the same specimen disclosed Gram-negative diplococci. Forty-five cubic centimeters of cloudy spinal fluid, found to contain extra- and intracellular Gram-negative diplococci, were removed at 10.00 p.m. and replaced by 30 cc. of Lederle's antimeningitic serum; and at the same time 30 cc. of Mulford's antimeningitic serum were administered subcutaneously.

The following morning the patient was taken to the Jewish Hospital, where 50 cc. of cerebrospinal fluid were withdrawn and 30 cc. of antimeningitic serum obtained from The Rockefeller Institute injected

intrathecally and 110 cc. intravenously. That same night 60 cc. of spinal fluid were withdrawn and 30 cc. of Rockefeller serum introduced into the spinal canal. Kernig's sign was first observed that morning and Brudzinski's head and leg signs that night. Vigorous treatment was continued, in all 430 cc. of serum being administered. Three days later the first mental impairment occurred—a slight flightiness; with somnolence and Cheyne-Stokes respiration the following morning, and respiratory paralysis and death in the afternoon. On this day 60 cc. of clear spinal fluid were withdrawn and found to contain many meningococci. Specimens of spinal fluid removed three and four days previously had been examined by Dr. John A. Kolmer, Dr. Herbert Fox and Dr. Edward A. Steinfield, all of whom failed to find meningococci. Three days before the patient's death, swabs from his pharynx, tonsils and rhino-pharynx were smeared lightly on the bottom of a sterile test tube and overlaid with 10 cc. of his whole, coagulable blood—according to the method described by Myer Solis-Cohen and Heist (10)—by Louis S. Borow. After twenty-four hours' incubation there grew up a Gram-negative diplococcus, fulfilling the criteria for the meningococcus in growth and morphology, and agglutinated by Flexner's polyvalent serum. On the day preceding death the white blood cell count was 8000, polynuclears 87 per cent, large mononuclears 3 per cent, lymphocytes 10 per cent. Since the second day of the illness the highest temperature had been 99.2° F., the highest pulse 64, and the highest respiratory rate 24.—S. S. C. and M. S. C.

CONCLUSIONS

1. When meningococci, freshly isolated from the *spinal fluid* of a patient with cerebrospinal meningitis, are cultured in capillary tubes of the whole, coagulable blood of normal men, they are found to possess an ability to grow rapidly in that medium. This ability is not possessed by the majority of the strains of meningococci freshly isolated from the *throats of carriers*. Experiment has proved that there is a correlation between the ability of meningococci (as well as certain other bacteria) to grow rapidly in whole, coagulable blood and their virulence for the species from which the blood was taken. We are led to conclude that the spinal fluid strains of meningococci are much more virulent for man than are the carrier strains.

2. Certain *carrier strains* grow better in whole, coagulable human blood than do others. We conclude that they are the more virulent for man. The majority of carrier strains are relatively low in virulence or non-virulent.

3. The whole, coagulable blood of most normal men will permit the rapid growth of *spinal-fluid* strains. This indicates that most men are susceptible to the attacks of meningococci that have passed through the human nervous system.

4. The blood of but *one among many normal men* permits the rapid growth of *carrier* strains. This minority of men is more likely to develop meningitis after exposure to a carrier. It is probably among this group that most of the cases of *meningitis* occur.

REFERENCES

(1) Heist, George D., Solis-Cohen, Solomon, and Solis-Cohen, Myers The bactericidal action of whole blood, with a new technique for it: determination. Jour. Immunol., 1918, **3**, 261.

(2) Bull, Carroll G., and Bartual, Luis: Pneumococcus cultures in whole fresh blood. 1. The retardative effect of the blood of immune animals, and the mechanism of the phenomenon. Jour. Exper. Med., 1920, **31**, 233.

(3) Matsunami, Taitsu, and Kolmer, John A.: The relations of the meningococcidal activity of the blood to resistance to virulent meningococci. Jour. Immunol., 1918, **3**, 201.

(4) Matsunami, Taitsu: Studies on the meningococcidal activity of blood. Jour. Immunol., 1920, **5**, 51.

(5) Black, J., Fowler, Kenneth, and Pierce, Paul: Development of the bactericidal power of whole blood and antibodies in serum. Jour. Amer. Med. Asso., 1920, **75**, 915.

(6) Solis-Cohen, S., Heist, George, D., and Solis-Cohen, Myer: Observations on the behavior of diphtheria bacilli in whole, coagulable blood, with a comparison of the results of the tests for bactericidal and antitoxic immunity in the same persons. Trans. Asso. American Phys., 1920, **35**, 263.

(7) Heist, George D., and Solis-Cohen, Solomon: The bactericidal action of the whole blood of rabbits following inoculations of pneumococcus bacterins. Jour. Immunol., 1919, **4**, 147.

(8) Solis-Cohen, Myer, Heist, George D., and Borow, Louis S.: The reaction of diphtheria-like bacilli with the fresh, whole, coagulable blood of an animal susceptible to diphtheric infection. (Read before a joint meeting of the American Association of Immunologists with the American Association of Pathologists and Bacteriologists at Cleveland, Ohio, March 25, 1921, and as yet unpublished.)

(9) Medical War Manual, No. 6. Phila., 1919, page 49.
(10) Solis-Cohen, Myer, and Heist, George D.: A method of distinguishing from among various microorganisms present in a patient those that are and those that are not acted upon by that patient's whole blood. Penna. Med. Jour., Oct., 1921, **25**, 27.

THE ACTION OF VARIOUS METALLIC SALTS ON HEMOLYSIS

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During a series of investigations concerning the formation conditions for various bacterial toxins, one of us (W.) made the observation, that the presence of quite negligible quantities of various metallic salts in the substratum often had a rather conspicuous effect on the amount of toxin formed in the cultures.

These experiments, which were performed with staphylolysin and diphtheria toxins (1), showed that this action, to a great extent, depended on the concentration of the metallic salt; a certain concentration exerted a maximal effect, the action diminishing with increasing as well as with decreasing concentrations.

This observation, which is of a certain theoretical interest and which, moreover, will probably become of practical value in connection with the preparation of diphtheria toxin, induced us to undertake an investigation—necessary for the estimation of the results—of the sensitivity of the blood-corpuscles employed in the staphylolysin experiments towards small quantities of certain metallic salts.

During the first of these investigations the metallic salts were observed in some cases to augment and in other cases to reduce the resistance of the blood-corpuscles to the staphylolysin, and this often in a very considerable degree. Therefore, we thought it would be of interest to enter into a more thorough investigation of these relations, and we extended the experiments to comprise besides staphylolysin-goats' blood-corpuscles, also saponin-horses' blood-corpuscles, and complement-amboceptor-sheep blood-corpuscles.

Such investigations should of course be performed in such a way that the action of the individual salts be examined in many concentrations from just the minimal dose that produces a demonstrable reaction up to the maximal dose applicable at all; as most metallic salts produce either hemolysis, agglutination or discoloration of the suspension of blood-corpuscles, there will always be a limit to the quantity of such substances that may be employed for experiments of this kind (see table1). We have,

TABLE 1

SALTS	CONCENTRATION OF SOLUTION	HORSES' BLOOD-CORPUSCLES 8 cc.—1 PER CENT	GOATS' BLOOD-CORPUSCLES 8 cc.—1 PER CENT	SHEEP'S BLOOD-CORPUSCLES 1 PER CENT	
				8 cc.	1.25 cc.
$LiCl$	$\frac{1}{1}$ molecular	0.2	0.2	0.2	0.03
$CaCl_2$	molecular	0.2	0.2	0.2	0.03
$Sr(NO_3)_2$	molecular	0.2	0.2	0.2	0.03
$Ba(NO_3)_2$	$\frac{1}{3}$ molecular	0.2	0.2	0.2	0.03
$Pb(C_2H_3O_2)_2$	$\frac{1}{2}$ molecular	0.0003	0.0003	0.0003	0.00005
$BeCl_2$	$\frac{1}{1}$ molecular	0.0002	0.0003	0.0003	0.00005
$MgSO_4$	molecular	0.2	0.2	0.2	0.03
$ZnSO_4$	molecular	0.0001	0.0003	0.00025	0.00004
$CdCl_2$	molecular	0.005	0.005	0.003	0.0005
$CuSO_4$	molecular	0.00001	0.0005	0.00006	0.00001
$NiCl_2$	molecular	0.0035	0.015	0.003	0.0005
$Co(NO_3)_2$	molecular	0.005	0.005	0.003	0.0005
$FeCl_3$	molecular	0.00001	0.00004	0.00012	0.00002
$MnSO_4$	molecular	0.2	0.2	0.1	0.015
$CrCl_2$	molecular	0.00003	0.00004	0.0003	0.00005
$Al_2(SO_4)_2$	$\frac{1}{10}$ molecular	0.0001	0.001	0.0012	0.0002
$AgNO_3$	molecular	0.0005	0.0005	0.00025	0.00004
$HgCl_2$	molecular	0.0001	0.0001	0.00006	0.00001
$HCl \cdot AuCl_3$	molecular	0.00005	0.00004	0.00012	0.00002
$2HCl \cdot PtCl_4$	molecular	0.025	0.015	0.025	0.004

however, been obliged to confine our investigations of these relations to the two extremes. It is of course possible that several optimal or minimal concentrations may be found within these limits. In those cases, however, in which we have watched the progress through many concentrations, the action gradually diminished with decreasing doses. The actions on complement of Hg, Mg and Ag show, however, an inciting effect at one concentration and an inhibitive effect at another (see table 4).

The technic applied is described in connection with the individual experiments. The method employed for the hemolytic tests is the one usually employed at this institute, described by Madsen (2).

In a series of experiments we first determined the maximal dose of the various solutions of metallic salts which, without causing perceptible alterations (hemolysis, agglutination or discoloration), might be added to the volume of blood-corpuscles employed for the experiments. The results of these determinations are shown in table 1.

In the case of sheep's blood-corpuscles the permissible quantity is stated for 8 cc. of blood for the sake of comparison and also for 1.25 cc. which is the quantity employed in the following experiments. The blood-corpuscles were washed as stated in the preliminary accounts of the respective experiments.

No larger doses than 0.2 cc. of the molecular solutions have been employed.

As appears from table 1, the sensitivity of the blood-corpuscles towards the various metallic salts is extremely variable, nor does there seem to be any homogeneity within the various groups of metals. The figures stated are of course not absolute, but may vary somewhat in the experiments performed at different times, presumably chiefly owing to a varying content of serum in the suspension.

ATTEMPTS AT DETERMINING THE MINIMAL CONCENTRATION OF THE RESPECTIVE METALLIC SALTS, AT WHICH THEIR ACTION IS MEASURABLE

These experiments are performed in the following manner: To a series of test-tubes containing decreasing doses of the metallic salt, hemolysin is added in such a quantity as to produce only a slight hemolysis (about 20 to 30 per cent); after filling up with a physiological solution of sodium chloride to the volume stated, the blood-corpuscle suspension is added, this mixture being then, after shaking, placed in the thermostat and afterwards in the ice-box overnight. The next day a determination is made of how much hemoglobin (in per cent) has been dissolved in each tube.

EXPERIMENTS WITH SAPONIN

For the experiments with saponin, horses' blood-corpuscles have been employed; the defibrinated blood was centrifugalized and washed twice in a 0.9 per cent solution of sodium chloride, and from these washed corpuscles was prepared a 1 per cent suspension, to which was added 2 per cent of a 2 per cent solution

TABLE 2

Saponin; horses' blood-corpuscles

	SALTS	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	AVERAGE	RECIPROCAL VALUES
Favoring hemolysis	$AuCl_3$	0.0035	0.0031	0.0035	0.0034	294.0
	$HgCl_2$	0.007	0.0065	0.0065	0.0067	149.0
	$AgNO_3$	0.04	0.037	0.04	0.039	25.6
	$Pb(C_2H_3O_2)_2$	0.1	0.08	0.1	0.09	11.1
	$Co(NO_3)_2$	0.5	0.55	0.5	0.52	1.92
	$MnSO_4$	2.0	2.5	1.8	2.1	0.476
	$Ba(NO_3)_2$	7.0	10.0	8.0	8.0	0.125
	$MgSO_4$	9.0	10.0	10.0	9.7	0.103
	$CaCl_2$	10.0	10.0	12.0	10.7	0.094
	$Sr(NO_3)_2$	10.0	13.0	13.0	12.0	0.083
No effect	$LiCl$					
	$BeCl_2$					
	$PtCl_4$					
Inhibiting hemolysis	$CdCl_2$	4.0	5.0	4.0	4.3	0.233
	$NiCl_2$	2.3	2.5	2.5	2.4	0.417
	$ZnSO_4$	0.07	0.075	0.08	0.075	13.3
	$Al_2(SO_4)_3$	0.008	0.0085	0.008	0.0081	123.0
	$CuSO_4$	0.007	0.007	0.008	0.0073	137.0
	$FeCl_3$	0.006	0.006	0.0058	0.006	167.0
	$CrCl_2$	0.005	0.0045	0.005	0.0048	208.0

of citrate of sodium in order to prevent spontaneous agglutination (Atkin (3)).

The mixture of metallic salts and saponin was diluted to 2 cc. by means of a physiological solution of sodium chloride, and 8 cc. of the 1 per cent blood-corpuscle suspension were added. The mixtures were kept in the water-bath at 37°C. for one hour, and then, after repeated shaking, in the ice-box overnight. It could

now be very readily ascertained how large a concentration of salt was necessary to exert an action; whether this be promotive or inhibitive of hemolysis.

At different times and employing different specimens of blood, three experiments were performed, the results of which are collated in table 2.

The figures stand for the minimum concentration of metallic salt in cubic millimetre amounts of $\frac{1}{1}$ molecular solution, at which an action is demonstrable. For the sake of comparison the reciprocal values of the average figures are stated in the last column.

EXPERIMENTS WITH STAPHYLOLYSIN

For these experiments goats' blood-corpuscles were employed, prepared in quite the same way as the horses' blood-corpuscles for

TABLE 3

Staphylolysin; goats' blood-corpuscles

	SALTS	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	AVERAGE	RECIPROCAL VALUES
Favoring hemolysis	$AuCl_3$	0.0031	0.003	0.0029	0.003	333.0
	$HgCl_2$	0.007	0.0064	0.0061	0.0065	154.0
	$Co(NO_3)_2$	0.02	0.02	0.017	0.019	52.6
	$AgNO_3$	0.035	0.03	0.031	0.032	31.3
	$MnSO_4$	0.045	0.04	0.04	0.042	23.8
	$PtCl_4$	0.1	0.08	0.08	0.09	11.1
	$NiCl_2$	0.15	0.12	0.13	0.13	7.69
	$MgSO_4$	0.16	0.15	0.16	0.16	6.25
	$CdCl_2$	0.23	0.2	0.2	0.21	4.76
	LiCl	170.0	100.0	150.0	140.0	0.007
Inhibiting hemolysis	$Sr(NO_3)_2$	5.0	5.5	5.5	5.3	0.189
	$Ba(NO_3)_2$	4.1	4.0	4.5	4.2	0.238
	$CaCl_2$	2.5	3.1	3.0	2.9	0.345
	$ZnSO_4$	0.15	0.12	0.17	0.15	6.67
	$Pb(C_2H_3O_2)_2$	0.12	0.13	0.13	0.13	7.69
	$CuSO_4$	0.1	0.12	0.11	0.11	9.09
	$Al_2(SO_4)_3$	0.07	0.07	0.085	0.075	13.3
	$BeCl_2$	0.05	0.05	0 05	0.05	20.0
	$CrCl_2$	0.03	0.03	0.032	0.031	32.3
	$FeCl_3$	0.03	0.027	0.03	0.029	34.5

The figures are to be understood as in table 2.

the saponin experiments, the other experimental procedures being also identical. While the horses' blood-corpuscles are dissolved by the saponin when the mixtures are standing in the thermostat at 37°C., hemolysis of the goats' blood-corpuscles with staphylolysis does not occur until during the ensuing cooling process (Walbum (4)). In order to avoid zones of different color shades developing in the test tubes used for hemolysis, which will readily occur as a consequence of the sedimentation of the blood-corpuscles during the hemolytic action, the mixture on removal from the water-bath at 37°C. is well shaken and cooled for thirty minutes in cold water (about 10°C.) and then, again shaken and placed in the ice-box.

The results of the three experiments are to be found in table 3.

EXPERIMENTS WITH COMPLEMENT-AMBOCEPTOR

These experiments were performed with sheep's blood-corpuscles and the corresponding amboceptor produced by injection of guinea-pig's kidney into rabbits; as complement fresh guinea-pig's serum was employed.

The experiments were carried through with a total volume of 1.25 cc.; 1 per cent sheep's blood-corpuscles, the amboceptor volume, found by means of titration, and a complement volume producing a hemolysis of about 25 per cent. The metallic salt solution was first mixed with complement plus the solution of physiological sodium chloride (for correction of volume) after which the blood-corpuscles, sensitized by amboceptor were added. The tubes were placed one hour in the water-bath at 37°C., and overnight in the ice-box.

The results of these three experiments performed at different times, are collated in table 4.

It appears from tables 2, 3 and 4, that the action of the metallic salts on the hemolytic agents varies considerably, not only quantitatively but also qualitatively. The agreement between the individual experiments with each hemolysin is surprisingly close but this is dependent upon very careful treatment of the blood-corpuscles during centrifugalization and washing.

While Co in all cases exerts a promotive action on hemolysis and Fe, Cr, Al, Cu and Zn an inhibitive one, the effect of Hg, Ag and

TABLE 4

Complement-amboceptor; sheep blood corpuscles

	SALTS	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	AVERAGE	RECIPROCAL VALUES
Favoring hemolysis	$NiCl_2$	0.005	0.005	0.0055	0.0052	192.0
	$Co(NO_3)_2$	0.005	0.0053	0.0052	0.0052	192.0
	$HgCl_2$	0.005	0.0052	0.0055	0.0052	192.0
	$MgSO_4$	0.005	0.0055	0.0055	0.0053	189.0
	$AgNO_3$	0.01	0.008	0.008	0.0087	115.0
	$LiCl$	0.5	0.45	0.45	0.47	2.13
Inhibiting hemolysis	$MgSO_4$	16.0	16.0	19.0	17.0	0.558
	$CaCl_2$	1.5	1.7	1.6	1.6	0.625
	$Sr(NO_3)_2$	1.0	1.2	1.5	1.2	0.833
	$Ba(NO_3)_2$	0.8	0.75	0.78	0.78	1.28
	$CdCl_2$	0.08	0.085	0.08	0.082	12.2
	$AgNO_3$	0.03	0.033	0.035	0.033	30.3
	$BeCl_2$	0.032	0.033	0.032	0.032	31.3
	$CrCl_2$	0.03	0.032	0.03	0.031	32.3
	$HgCl_2$	0.02	0.018	0.023	0.02	50.0
	$Pb(C_2H_3O_2)_2$	0.015	0.013	0.015	0.014	71.4
	$Al_2(SO_4)_3$	0.01	0.009	0.01	0.0097	103.0
	$FeCl_3$	0.0075	0.0067	0.0083	0.0075	133.0
	$CuSO_4$	0.0055	0.0052	0.0055	0.0054	185.0
	$ZnSO_4$	0.005	0.0048	0.0055	0.0051	196.0
	$MnSO_4$	0.005	0.0045	0.0045	0.0047	213.0
	$PtCl_4$	0.0005	0.00052	0.00055	0.00052	1920.0
	$AuCl_3$	0.0004	0.00042	0.0004	0.00041	2440.0

The figures are to be understood as in table 2.

TABLE 5

METALS	SAPONIN	STAPHYLOLYSIN	COMPLEMENT-AMBOCEPTOR
Co	+	+	+
Hg	+	+	+÷
Ag	+	+	+÷
Mg	+	+	+÷
Au	+	+	÷
Mn	+	+	÷
Ni	÷	+	+
Li	0	+	+
Pt	0	+	÷
Ca	+	÷	÷
Ba	+	÷	÷
Sr	+	÷	÷
Cd	÷	+	÷
Pb	+	÷	÷
Zn	÷	÷	÷
Al	÷	÷	÷
Cu	÷	÷	÷
Cr	÷	÷	÷
Fe	÷	÷	÷
Be	0	÷	÷

+ Means favoring, ÷ inhibitive of, and 0 no action on hemolysis.

Mg, which all acted favorably on hemolysis in the saponin and staphylolysin tests, assumed two forms in the complement test, the salts of these metals acting favorably at a certain concentration and inhibitively at another concentration.

The effect of the other metallic salts tested is summarized in table 5.

Whether this action of the salts may be due to a direct influence on the hemolytic agent (for instance a destructive action on the complement) or to the blood-corpuscles, will rest with future investigations to ascertain.

EXPERIMENTS WITH THE MAXIMAL CONCENTRATIONS OF THE VARIOUS METALLIC SALTS THAT CAN BE APPLIED WITHOUT PRODUCING HEMOLYSIS, AGGLUTINATION OR DISCOLORATION OF THE BLOOD CORPUSCLES

These experiments are so performed that the respective hemolytic agents are employed in such quantities as to produce only a partial hemolysis; the doses of the various metallic salts being as those stated in table 1. For each experiment the hemolysin was pipetted off in 3 to 5 different doses (all giving partial hemolysis but, of course, in a different degree), to this was added the solution of the metallic salt and a solution of physiological sodium chloride up to a definite volume and, finally, the blood-corpuscle suspension; otherwise the technic was quite identical to what has been previously described.

By means of a control series of hemolysin and blood-corpuscles alone, the percentage augmentation was calculated for each mixture that allowed of such calculation, that is, each mixture in which total hemolysis did not occur.

From the 3 to 5 figures thus obtained for each experiment and for each metallic salt, the average augmentation percentage was calculated.

Of such experiments, four with saponin-horses' blood-corpuscles, five with staphylolysin-goats' blood-corpuscles, and five with complement-amboceptor sheep blood-corpuscles were performed at different times. The results of all these investigations are stated in tables 6, 7 and 8, the figures thus stating the average

TABLE 6

Saponin; horses' blood-corpuscles

SALTS	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III	EXPERIMENT IV	AVERAGE AUGMENTATION
					per cent
Li	20	40	57	25	36
Ca	2233	1900	1900	1300	1833
Sr	617	233	900	140	473
Ba	75	33	213	108	107
Pb	13	90	19	0.7	30.7
Be	13	57	÷4	13	20
Mg	617	167	150	140	260
Zn	53	74	132	46	76
Cd	31	47	13		30
Cu		57	23	÷4	25
Ni	20	47	0	÷7	15
Co	27	27	13	128	49
Fe		74	÷7	18	28
Mn	484	567	650	500	550
Cr		57	56	18	44
Al	0	74	21	29	31
Ag	13	33	29	43	30
Hg	104	200	67	219	148
Au		57	7	35	33
Pt	42	44	38	47	43

The figures state average augmentation in per cent.

TABLE 7

Staphylolysin; goats' blood-corpuscles

SALTS	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III	EXPERIMENT IV	EXPERIMENT V	AVERAGE AUGMENTATION IN PER CENT
Li	÷6	48	83	÷10	5	24
Ca, Sr	Total abolition of the action of the lysin					÷100
Ba	÷98		÷66	÷100	÷100	÷91
Pb	÷71	4	÷79	÷40	÷53	÷48
Be	÷82	÷35	÷97	÷72	÷88	÷75
Mg	1629			40	37	569
Zn	1107	543		80		577
Cd	450	44	335	20	100	190
Cu	÷76	÷66	÷87	÷76	÷63	÷74
Ni, Co	The augmentation cannot be calculated from the experiments, but is sure to be over 2000 per cent					>2000
Fe	÷46	176	÷58	30	÷67	7
Mn	1629			40	54	574
Cr	÷87	÷88	÷100	÷50	÷95	÷84
Al	÷83	÷81	÷92	÷96	÷91	÷89
Ag	98	50	117	÷30	÷64	34
Hg	184	71	÷13	÷30	÷38	35
Au	÷29	71	÷41	÷20	÷59	÷16
Pt	117	106	460	60	64	161

augmentation in percentage for the individual experiments and, in the last column of the tables, the average augmentation for all the experiments taken together.

While the previous experiments, in which the purpose was to determine the minimal active dose of the metallic salts, showed very close congruity in spite of their being performed at

TABLE 8

Complement-amboceptor; sheep's blood-corpuscles

SALTS	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III	EXPERIMENT IV	EXPERIMENT V	AVERAGE AUGMENTATION IN PER CENT
Li	55			62	32	50
Ca	÷100			÷94	÷100	÷98
Sr	÷100			÷100	÷100	÷100
Ba	÷86			÷47	÷79	÷71
Pb	÷32	÷3	6	÷6		÷9
Be	÷17	0	0	÷6		÷6
Mg	÷100			÷49	÷98	÷49
Zn	÷71	÷52	÷75	÷73		÷68
Cd	÷91	÷73	÷88	÷73		÷81
Cu	÷18	÷16	÷10	÷24		÷17
Ni	69	74	29	62		59
Co	÷2	0	÷25	÷16		÷11
Fe	÷2	÷6	0	÷16		÷6
Mn	÷90	÷74	÷74	÷94		÷83
Cr	14	0	16	÷1		7
Al	18	0	13	12		11
Ag	÷10	0	15	3		2
Hg	÷10	÷10	0	÷24		÷11
Au	2	3	5	÷4		2
Pt	÷91	÷32	÷69	÷56		÷62

different times, there is a very conspicuous variation in these latter experiments. As the only varying factor in the experiments is a difference of the concentration at which the metallic salts were employed, the cause of the incongruity should presumably be sought therein; it seems as if blood-corpuscles, to which is bound as large a quantity of a metallic salt as can be bound without acting in a perceptibly destructive manner acquire strongly varying stability towards various hemolytic agents.

The considerable disagreement, which is found almost throughout, between these latter experiments and those previously described in this article, should probably be assigned to relations of this kind.

We have made several experiments in order to determine whether the action of these metallic salts may be due to the cation or to the anion. These experiments were performed with chlorides, sulphates and nitrates of Mg, Mn, Zn and Ni; it was ascertained in all cases that the anion was without significance in this respect.

SUMMARY

What has been examined in this study is the significance of the presence of small quantities of metallic salts on the hemolytic action of saponin on horses' blood-corpuscles, of staphylolysin on goats' blood-corpuscles, and of complement-amboceptor on sheep blood-corpuscles. By determining the minimal dose of the individual salts (in molecular solution) at which their action is demonstrable, it is possible to obtain a comparison between the action of the different salts. While some salts exert an inciting action on hemolysis, others exert an inhibitive one (positive and negative catalysis?); some show an inciting effect at one concentration and an inhibiting one at another (table 5).

On examining blood-corpuscles to which is bound as large a quantity of a metallic salt as can be bound thereto without acting perceptibly destructively, the stability of such blood-corpuscles towards the action of hemolytic substances will be found to vary greatly, to which fact the considerable experimental inconstancy in the experiments described should probably be assigned.

The anion in the salts seems to be without significance as regards their action in either favoring or inhibiting hemolysis.

REFERENCES

(1) Walbum, L. E.: Compt. rendus des séances de la Soc. de Biologie, Paris, 1921, **85**.

(2) Madsen, Th.: Kraus and Levaditi; Handbuch . . . d. Immun., Jena, 1908, **1**, 35–144.

(3) Atkin, E. E.: Zeitschr. f. Immunitätsforschung, Orig., 1909, **1**, 387.

(4) Walbum, L. E.: Zeitschr. f. Immunitätsforschung, Orig., 1909, **3**, 70.

AN ALLERGIC REACTION OF THE TUBERCULOUS UTERINE HORN

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The reactivity of the uterus of the sensitized guinea-pig in the anaphylactic experiment has received much study and has contributed greatly to the understanding of the mechanism of specific allergic phenomena. The experiments recorded here are simply an application of the principle of specific reactivity between antigen and its homologous antibody, employing the sensitive uterus as an indicator.

Immediately after the publication by Weil (1) in 1917 of his results upon the reaction of the tuberculous guinea-pig uterus to Old Tuberculin, experiments were started with the idea of using this method to detect tubercle protein or specific products of tubercular metabolism in the urine of cases of tuberculosis. Such experiments were based in part upon the results of Blake (2) in reporting specific precipitinogen in the urine in pneumonia, and in part upon the intimate relationship which had been shown to exist between precipitin and anaphylactic antibody by Weil and others. It seemed logical to assume that tubercle protein might be eliminated through the kidneys in a manner similar to that demonstrated for pneumococcus protein in pneumonia. Also, from the experiments of Weil it was evident that in the tuberculous guinea-pig the uterine musculature shares in the general allergic condition of the body and is capable of reacting to minute amounts of tubercle protein. Consequently, a series of tests were performed in which the uterine horn of the tuberculous pig was suspended by the Schultz-Dale method and to the bath solution, urine from different sources was added.

The reactions obtained were extremely varied in character. With supposedly normal urines as a rule no reactions were secured with the amounts of urine employed; with urines from cases reported to have tuberculosis clinically reactions were sometimes absent and at other times extremely sharp and analogous in every respect to the acute anaphylactic response.

Because of this variability in result, and because of the impossibility at that time of obtaining adequate data regarding the cases from which the urines were derived the work was discontinued, only to be resumed during the past few months. The immediate stimulus to continuing the experiments was afforded by the reports on the Wildbolz reaction (3) which have appeared during the past year. These findings offered additional support to the underlying principle since they demonstrated directly the presence of tubercle protein or of metabolic products in the urine capable of eliciting the cutaneous allergic phenomenon in sensitive individuals.

In the more recent experiments the urines have all been derived from cases in which acid-fast organisms—in most cases by inoculation proved to be *B. tuberculosis*—were demonstrated in the urine. The tests have been conducted by suspending a uterine horn from a tubercular guinea-pig and a horn from a normal guinea-pig in the same bath of oxygenated Locke solution. After relaxation of the horns and the appearance of the regular rhythmic contractions the urine from one of the cases was added to the bath solution (350 cc.), usually in 3 to 5 cc. amounts. In no instance has this amount of urine from any of the cases induced a marked reaction in the normal horn while in some cases the tubercular horn responded sharply as evidenced by the kymographic record. (Fig. 1.)

That the reaction of the tubercular horn was not simply an increased susceptibility to urine *per se* and was devoid of specific significance was shown repeatedly by adding to the bath solution first a normal urine, to which no response was secured, and then after washing and renewing the bath, adding a tuberculous urine with the manifestation of an immediate contraction of the horn from the infected animal. Reactions of the type described above

cannot be elicited with the smooth muscle of every infected pig, even though by experiment the urine can be shown to produce a reaction in certain animals. Obviously, during the course of tubercular infection there is a stage of anergia, or it is possible

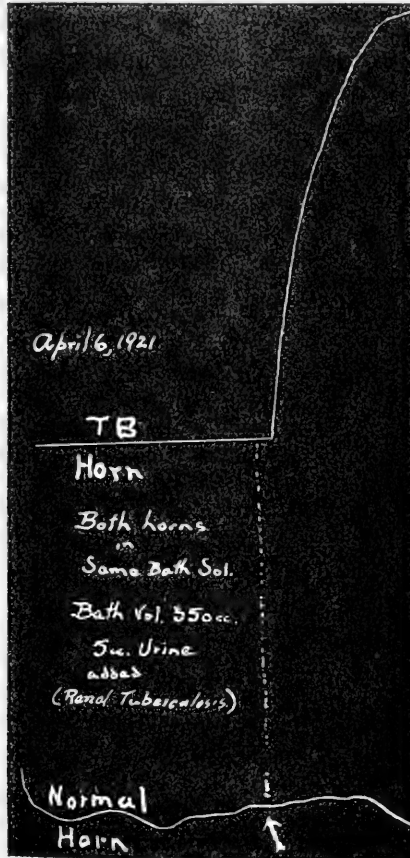

Fig. 1

certain animals never acquire a reactive state. This phase of the problem requires further investigation.

The question as to whether the reaction is a true anaphylactic response is not significant in this connection; although it may be said that the capacity for specific contraction is markedly diminished or absolutely abolished after a single maximal response.

This suggests a possible desensitization through specific antigen-antibody union.

The reactions described above have been conducted with but eight tubercular urines from known cases, and with seven of these, reactions of the tuberculous horn have been secured of greater or less intensity as compared with normal tissue.

Apparently any urine if used in sufficient amounts will throw any uterine horn into contraction; thus it is essential that quantitative factors be observed. In the experiments of this series this non-specific factor was controlled by having the tuberculous horn and the normal horn in the same bath where they were equally subject to non-specific stimulation.

The possibility that the reaction may be of diagnostic value presents itself, but as yet the amount of work done does not warrant such a conclusion.

REFERENCES

(1) Weil: *Jour. Am. Med. Assn.*, 1917, **68**, 972.
(2) Blake: Monograph No. 7, Rockefeller Institute, 1917, 30.
(3) Wildbolz: Cor.-Bl. f. schweiz. Aerzte, 1919, **49**, 793; Schweiz. med. Wchnschr. 1920. 1, 32.

RELATIONSHIP OF VARIOUS ANTIORGAN SERA

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In our previous studies (1, 2) concerning tissue specificity we were constantly faced with the fact that the various tissues showed a very great complexity of relationship. In many cases this complexity tended to cloak the evidence of the tissue specificity and it was only by means of comparative, quantitative studies that we were able to prove certain facts. It was, however, evident that tissue specificity could actually be demonstrated in the case of the tissues which were used (namely, liver, kidney, spleen, brain, muscle and testicle). Furthermore, there was noted a relationship between the various organs of animals of the same species, which was evidently due to a species specificity. In addition there were suggestions of a further relationship between the various organs which could not be explained by the simple species specificity. It was noted that when we absorbed an antiserum with a non-homologous tissue and tested the complement fixing power of the serum with the antigen corresponding to the absorbent, there became evident a relationship between the absorbent and the antigen shown by the apparently greater absorption of the antibodies under these circumstances. This phenomenon did not appear constantly nor in the case of all sera and all absorbents, but it appeared with sufficient regularity in certain sera to be worthy of further investigation. It did, as stated above, serve in many cases to confuse the results and make more difficult their interpretation. It must, of course, be borne in mind that we used in our experiments, not a portion, or chemical derivatives of the various organs but the entire organ; thus were included all of the complex chemical substances which

might be contained therein. It was neither our intention nor desire to work with a limited number of constituents of, or a purified chemical substance obtained from the organ, but rather to determine the immunological relations of the whole tisssue both to identical tissues and to other tissues. This phenomenon of the relationship between absorbent and antigen and the suggestion of the certain more specific relationships between the various tissues might, it appeared, be due to one of three causes or even a combination of these causes:

1. Certain tissues might be in general better absorbents than other tissues and might thus give rise to this apparent relationship between absorbent and corresponding antigen in certain cases; this type of reaction would not completely correspond to the facts, however, since the absorbent-antigen relationship was apparent with practically all the tissues. We shall, however, discuss this relationship again.

2. It is possible that there exists a closer immunological relationship between certain organs and tissues than between others, which permits, therefore, a greater absorption by certain tissues from certain antisera and which might give us the results mentioned above.

3. Finally, there exists the possibility that each organ or tissue contains antigens, chemical substances or structures which are more or less identical with the structure of antigens contained in and composing the principal or chief antigen of other organs or tissues. Thus kidney tissue might contain not only the specific kidney antigen (and of course the antigen corresponding to the general species specific substance) but also chemical structures more or less identical with the preponderant and specific substance of liver or brain or spleen.

These three possibilities, if actually existent, might each singly give us the apparent absorbent-antigen relationship; they might act singly to give this apparent result, or any two of them, or even all three acting together, might produce this apparent phenomenon.

Our problem was therefore to analyse the influence of those various potential factors in bringing about this reaction.

METHODS AND TECHNIC

The basis of our experiments was the absorption of the various antiorgan sera with the various tissues and the subsequent testing of the complement fixing power of the sera with various tissue antigens. These steps and methods have been fully described in an earlier article and we shall therefore not repeat them here. However, in order to determine the relations of the various tissues we carried out what we shall, for convenience sake, call "combined" absorptions—that is, we brought the antisera into contact with two different types of tissue, serving as absorbents, previous to carrying out the complement fixation reactions. Thus we absorbed kidney serum with not only guinea-pig kidney, liver, brain or corpuscles singly, but also with combinations of these tissues such as kidney and liver, kidney and brain, kidney and corpuscles, liver and brain, etc. By then carrying out complement fixation reactions with various antigens and comparing the antibodies which had apparently been absorbed, we were able to determine whether the "combined" absorption removed more than did absorption with a single tissue. The actual quantity of tissues used as absorbents was in each case a unit, corresponding approximately to one quarter the volume of the serum; thus when we carried out the combined absorption we actually brought the serum into contact with double the unit volume of tissue. To serve as a check upon this increased amount of absorbent we also tested the action or influence of two unit volumes of each of the tissues. We used kidney, liver and brain antisera; the four absorbents mentioned above: kidney, liver, brain and corpuscle[1] antigens. The majority of our experiments were carried out with the antikidney serum but confirmatory experiments were also carried out with the antibrain and antiliver sera, which in general corresponded with those of the antikidney serum. We shall therefore limit our discussion principally to a consideration of the results obtained with the kidney serum.

We shall not present the complete tabulation of our results as we do not believe that this would in any way serve to enforce the

[1] The corpuscle antigen was a 2.5 per cent suspension of washed guinea-pig red blood cells.

facts which have been demonstrated, and a detailed discussion of such a tabulation would only increase unduly the volume of this discussion. We therefore add at the end of this article several selected protocols to which we shall refer in the text.

DISCUSSION OF EXPERIMENTS

In trying to determine the validity of the three potential explanations of the appearance of the absorbent-antigen relations, we found that many of the experiments carried out could not be applied; thus in those experiments where there existed a relationship between antiserum and absorbent or a relationship between antigen and absorbent, certain definite and previously demonstrated relationships became evident and we could not progress by means of these comparisons. On the other hand these experiments which were not suitable for our immediate purposes served well as a check on our previous work and as controls to the other more immediately applicable experiments.

1. We were able to demonstrate, as we had previously, the relationship of antiserum and homologous absorbent; thus tissue specificity. This appears with kidney, liver and brain antisera in protocols 1, 2, 6, 7 and 10. We could also demonstrate the relationship of absorbent and corresponding antigen as shown in protocols 3 and 4. But with these non-homologous antigens (not related to the antiserum), it was not possible to demonstrate that the "combined" absorption was more effective in removing antibodies than was the single absorption with tissues corresponding to the antigen. Thus, for instance, using liver antigen and kidney antiserum we found the amounts absorbed by the combination of either liver and kidney or liver and brain to be no greater than the amounts absorbed by the liver alone; this appears from the experiments in protocols 3 and 4.

2. It was evident that in order to learn about the relationship of the absorbents it would be necessary to study particularly those experiments in which we dealt with an antigen homologous to the antiserum and with absorbents not corresponding to the antigen, or with an antigen which corresponded with none of the absorbents being considered. These conditions were, of course, fulfilled in the case of antikidney serum and kidney antigen when

tested after absorption with either liver or brain singly or combined; or when the same antiserum was used with corpuscle antigen and we determined the complement fixing power after absorption with kidney and brain singly and combined. This fact meant, of course, that only in certain special cases would these relationships become evident and that the particular conditions would of necessity vary with the different antisera.

In general the results and the general tendencies of the results are similar in the various experiments. Possibly the one factor which made most difficult the interpretation of the results was the variability of the absorbing power of individual specimens of similar tissues, and also to a smaller degree, slight variations in the results in different experiments, dependent possibly upon the individual antigens.

In the types of experiments mentioned above where as few homologous or corresponding factors as possible entered into consideration, and where we could leave out of account the definite absorbent-antigen relationship, it was found in many, but not in all cases, that the "combined" absorptions had removed more antibodies than had absorption by either one of the same tissues acting alone. All of the protocols, excepting 3 and 4, show these relations. In those cases, shown in several of these experiments, where this increased effect of the "combined" absorption did not appear, of course one or both of the single absorbent tissues removed similar quantities of antibodies but no more than did the "combined" tissues. In those cases in which we used the antigen homologous to the antiserum and nonhomologous absorbents the results were distinctly more irregular than when we used an antigen not homologous to the antiserum and, as one of the absorbents, tissue corresponding to the antiserum. It is particularly these latter experiments which are of interest and aid the interpretation of our results.

3. In these experiments in which we used corpuscles as one of the absorbents, as in protocols 1, 2, 3, 4, 6 and 7, and as the other of the "combined" absorbents a tissue not corresponding to the antigen—thus with kidney antigen, liver tissue, or with liver or brain antigen, kidney tissue—we found usually that the

combined absorption removes more than does either of the single absorbents.

4. While, therefore, our results are not constant in one direction they are certainly distinctly suggestive. With the probability existing and strongly suggested that the composition of a particular tissue varies in each animal, it would scarcely be expected that the same organs from different animals would show constantly the identical quantitative absorbent, antigenic or antibody stimulating powers. We may consider the possibility that these differences noted above may be simply due to chance. However, it appears that the "combined" absorption is actually more complete than is the single absorption for the result is evident in more than 60 per cent of the possible cases. It may be suggested that the quantitative relations of the absorbent play a part, namely, that since the serum is brought into contact with larger amounts of absorbent material in the "combined" absorptions we might well expect larger amounts of antibodies to be removed. We have to a certain extent a check upon this possibility, since in a number of cases we exposed the serum to double the usual amount of a single tissue, either homologous or non-homologous to the antiserum, as in protocols 9, 10, 11 and 12, and we find as a rule that no greater quantities of antibodies are absorbed by the larger amounts of tissue. In very few cases—less than 20 per cent—did the larger amounts of absorbent remove more antibodies. This 20 per cent incidence we may believe to be due to chance; and therefore in all likelihood the 60 per cent occurrence of greater absorption by "combined" tissues lies well above what we should expect from mere experimental chance.

DISCUSSION

It is evident that in the methods used in these experiments it has been possible to demonstrate that absorption of an antiorgan serum by a combination of tissues removes more antibodies than does absorption by a single one of these tissues. It is true that this relation was not demonstrable in each one of the experiments in which it might possibly have appeared, but it does ap-

pear in a sufficient proportion of experiments to make it very nearly certain that this phenomenon is due to some factor more compelling than mere chance and it therefore seems evident that this can be accepted as a definite fact.

It is improbable that this selective absorption is due to the same factors which play a part in species specificity, that is, due to quantitative differences in the various tissues of this particular type of antigen acting as absorbent. For were this the case we should constantly find one type of tissue acting as the best absorbent; and this we do not find to be true, for this greater absorbing activity of the "combined" tissues is evident with various combinations of tissues. Furthermore this would not serve to explain the absorbent-antigen relationship.

It appears, therefore, that the explanation of the phenomenon must depend upon a qualitative rather than a quantitative variability of absorbing activity of the tissues, which is in some degree specific for each tissue. This specificity or individuality of each tissue may be dependent upon any of the three factors mentioned in the beginning of this article, namely, the better absorbent quality of any one or particular tissue; the existence of a relationship between certain organs permitting the one non-homologous tissue to react constantly more strongly with another particular tissue; or the existence in each organ of not only the particular homologous specific substances but also of substances specific (to a greater or less degree) with other organs.

It does appear that certain tissues are in general better absorbents than are others. In our earlier work it appeared quite definitely that this was the case with liver, which absorbed more actively than the other tissues. The present experiments have reinforced this fact, but aside from this being evident in liver there is little or no difference noted between the relative absorbent activity of the other organs. Possibly such differences do exist which are, however, so slight that they are not demonstrable by our methods. However, such variations in the general and non-specific absorbing activity of individual organs would not account for the evident greater absorption by combinations. We are considering here a non-selective absorption in which the

absorbents would remove essentially the same type of immune bodies, and this could hardly account for the facts. It is true that this might have some influence upon the variations noted but can scarcely completely account for the phenomenon.

We do not find in these experiments, nor in our previous ones, any clear evidence of specific or fixed relationship between any two types of tissue. If such relationship between any two types of tissues existed they should of course be reciprocal and should be quite constant. While we suggested in our earlier work the possibility of such relationship and did not at that time consider it possible to exclude its existence, it appears from the more recent results that no such constant or reciprocal relations can be demonstrated. It is true that liver used as absorbent with any antisera gives evidence constantly of the removal of large amounts of antibodies when corpuscles acted as antigen and there is therefore a suggestion of a relationship here; however, the absorbent action is not reciprocal and corpuscles as absorbent do not appear to have any peculiar or striking relationship to liver. It is possible that the explanation for this action of liver tissue lies in the fact that the liver tissue contains relatively larger quantities of corpuscles than any other organ, and we deal here with a somewhat disguised absorbent-antigen relationship depending really on the corpuscles. This fact may also account, at least in part, for the constantly greater absorbent activity of liver.

The existence of the third possible relation seems to be borne out by the evidence of the results. It is most likely that there exists in each tissue in addition to substances which are specific to the species and substances specific for the particular organ, also substances which are more or less identical with the tissue specific substances of other organs or tissues. We can base this conclusion upon two facts: first, the constantly appearing evidence of a definite absorbent-antigen relationship which certainly suggests the existence of this non-homologous but specific antibody in the various antisera; and secondly, the evidence brought out by the "combined" absorptions that the individual tissues apparently do not absorb from a certain anti-

serum the identical antibodies. We must have clearly in mind the fact that two tissues combined give evidence of absorbing more than does either one singly, provided we use as antigen a non-homologous tissue, that is, an antigen not corresponding to either absorbent. In view of our previous conclusions this can occur only if the antibodies absorbed by the two absorbents are of diferent natures. We must remember that we have excluded in our experiments the action of each individual tissue and also the possible greater absorbing action due to the use of larger quantities of tissue. It must mean, for instance, that brain acting as absorbent with kidney serum removes among other possible antibodies certain ones which are specific for brain or very closely related to the brain specific antibodies.

It seems probable that the amount of non-homologous but relatively specific antigen or absorbent in any tissue may well be variable—thus in different specimens of kidney tissue the amount of antigen related to brain would probably be different; the variability of our results would suggest such a conclusion. It is also possible that this non-homologous tissue specific antigen is not identical with the similar homologous tissue specific antigen but is only more or less closely related. In view of the complex structure of the many chemical substances present in the tissues and in view of the presumption that chemical structures are the basic factors underlying immunological differentiations, this incomplete relationship may exist. Substances resembling each other in their chemical structure may, of course, give very similar immunological reactions and the phenomenon which we have noted may possibly be based upon some such chemical similarity rather than a chemical identity.

While it therefore appears that there exist in each tissue these non-homologous tissue specific substances, it is quite probable that their action is frequently modified by the varying quantities of the species specific substances and by the various degrees of absorbent activity of various tissues.

It is evident, as would be expected as a result of the use of the entire organ in our experiments, that we have dealt with a complex antigen which stimulates the production of many different types

Protocol 1. Antikidney serum; kidney antigen

SERUM AND ABSORBENT	QUANTITY OF SERUM IN CUBIC CENTIMETERS											
	0.08	0.06	0.05	0.03	0.01	0.009	0.008	0.006	0.004	0.002	0.001	0.0005
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Kidney	++++	+++	+++	+	0	0	0	0	0	0	0	0
Liver	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0
Brain	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0
Corpuscle	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	0
Kidney, liver	++++	+++	+++	0	0	0	0	0	0	0	0	0
Kidney, brain	++++	+++	+++	0	0	0	0	0	0	0	0	0
Kidney corpuscle	++++	+++	+++	0	0	0	0	0	0	0	0	0
Liver, brain	++++	++++	++++	++++	+++	++	++	0	0	0	0	0
Liver, corpuscle	++++	++++	++++	++++	++++	++++	++++	++	0	0	0	0
Brain, corpuscle	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0

Protocol 2. Antikidney serum; kidney antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS													
	0.04	0.03	0.02	0.01	0.009	0.008	0.007	0.005	0.004	0.003	0.002	0.001	0.0007	0.0005
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	0
Kidney..	++++	++++	++++	++++	++++	+++	++	0	0	0	0	0	0	0
Liver	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0
Brain.....	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	0
Corpuscles.....	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Kidney, liver....	++++	+++	++++	++++	+++	++	0	0	0	0	0	0	0	0
Kidney, brain...	++++	++++	++++	++++	++++	+++	+	0	0	0	0	0	0	0
Kidney, corpuscles.....	++++	++++	++++	++++	+++	++	0	0	0	0	0	0	0	0
Liver, brain...	++++	++++	+++	++++	++++	++++	++++	++++	+++	+	0	0	0	0
Liver, corpuscles.....	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+	0	0	0
Brain, corpuscles.....	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0

Protocol 3. Antikidney serum; liver antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS												
	0.5	0.4	0.3	0.2	0.1	0.08	0.06	0.04	0.02	0.015	0.01	0.006	0.002
	++++	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0
Kidney	++++	++++	++++	++++	++++	++++	++	0	0	0	0	0	0
Liver	++++	+++	0	0	0	0	0	0	0	0	0	0	0
Brain	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0	0
Corpuscles	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0	0
Kidney, liver	++++	+++	0	0	0	0	0	0	0	0	0	0	0
Kidney, brain	++++	++++	++++	++++	++++	++++	0	0	0	0	0	0	0
Kidney corpuscles	++++	++++	++++	+++	+	0	0	0	0	0	0	0	0
Liver, brain	++++	+++	++	0	0	0	0	0	0	0		0	0
Liver, corpuscles	++++	++	0	0	0	0	0	0	0	0	0	0	0
Brain, corpuscles	++++	++++	++++	++++	++	++	0	0	0	0	0	0	0

Protocol 4. Antikidney serum; brain antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS									
	0.1	0.08	0.06	0.05	0.03	0.01	0.008	0.006	0.004	0.002
	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Kidney	++++	++++	++++	++++	+++	0	0	0	0	0
Brain	++++	++++	++++	+++	+++	0	0	0	0	0
Corpuscles	++++	++++	++++	++++	++++	++++	++++	++	0	0
Kidney, brain	++++	++++	++++	+	0	0	0	0	0	0
Kidney, corpuscles	++++	++++	+++	+	0	0	0	0	0	0
Brain, corpuscles	++++	++++	++++	++	0	0	0	0	0	0

Protocol 5. Antikidney serum; corpuscle antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS											
	0.5	0.3	0.2	0.1	0.09	0.08	0.06	0.05	0.04	0.03	0.02	0.01
	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	+	0
Kidney	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	+	0
Liver	++++	++++	++++	++++	++++	+++	++	+	+	0	0	0
Brain	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	+	0
Corpuscle	0	0	0	0	0	0	0	0	0	0	0	0
Kidney, liver	++++	++++	++++	++++	++++	+++	+++	++	+	0	0	0
Kidney, brain	++++	++++	++++	++++	++++	++++	+++	+++	++	0	0	0
Kidney corpuscle	0	0	0	0	0	0	0	0	0	0	0	0
Liver, brain	++++	+++	+++	0	0	0	0	0	0	0	0	0
Liver, corpuscle	0	0	0	0	0	0	0	0	0	0	0	0
Brain, corpuscle	0	0	0	0	0	0	0	0	0	0	0	0

Protocol 6. Antiliver serum; liver antigen

ABSORBENT	QUANTITY OF SERUM IN CUBIC CENTIMETERS											
	0.07	0.05	0.03	0.02	0.01	0.009	0.008	0.006	0.004	0.002	0.001	0.0008
	++++	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0
Kidney	++++	++++	++++	++++	++++	++++	+++	0	0	0	0	0
Liver	++++	++	0	0	0	0	0	0	0	0	0	0
Brain	++++	++++	++++	++++	++++	++++	++++	++++	++	0	0	0
Corpuscles	++++	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0
Kidney, liver	++++	++	0	0	0	0	0	0	0	0	0	0
Kidney, brain	++++	++++	++++	++++	++	0	0	0	0	0	0	0
Kidney, corpuscle	++++	++++	++++	++++	++++	++++	+++	0	0	0	0	0
Liver, brain	++++	++	0	0	0	0		0	0	0	0	0
Liver, corpuscle	++++	+++	0	0	0	0	0	0	0	0	0	0
Brain, corpuscle	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0	0

Protocol 7. Antiliver serum; liver antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS										
	0.1	0.07	0.05	0.03	0.01	0.009	0.008	0.006	0.004	0.002	0.001
	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0
Kidney	++++	++++	++++	++++	++++	++++	++++	+++	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0
Brain	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0
Corpuscle	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0
Kidney, liver	0	0	0	0	0	0	0	0	0	0	0
Kidney, brain	++++	++++	++++	++++	+++	0	0	0	0	0	0
Kidney, corpuscles	++++	++++	++++	++++	++++	++++	++++	0	0	0	0
Liver, brain	0	0	0	0	0	0	0	0	0	0	0
Liver, corpuscle	0	0	0	0	0	0	0	0	0	0	0
Brain, corpuscle	++++	++++	++++	++++	++++	++	0	0	0	0	0

Protocol 8. Antiliver serum; corpuscle antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS											
	0.1	0.08	0.05	0.03	0.02	0.01	0.009	0.008	0.006	0.004	0.002	0.001
	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0	0
Kidney	++++	++++	++++	++++	++++	++++	+	0	0	0	0	0
Liver	++++	++++	++++	++++	++++	0	0	0	0	0	0	0
Brain	++++	++++	++++	++++	++++	+++	0	0	0	0	0	0
Corpuscles	0	0	0	0	0	0	0	0	0	0	0	0
Kidney, liver	++++	++++	++++	0	0	0	0	0	0	0	0	0
Kidney, brain	++++	++++	++++	+	0	0	0	0	0	0	0	0
Kidney, corpuscle	0	0	0	0	0	0	0	0	0	0	0	0
Liver, brain	++++	++++	++++	++	0	0	0	0	0	0	0	0
Liver, corpuscle	0	0	0	0	0	0	0	0	0	0	0	0
Brain, corpuscle	0	0	0	0	0	0	0	0	0	0	0	0

Protocol 9. Antiliver serum; corpuscle antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS											
	0.1	0.08	0.06	0.05	0.03	0.02	0.01	0.009	0.008	0.006	0.004	0.002
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Kidney	++++	++++	++++	++++	++++	++++	++	0	0	0	0	0
Liver	++++	++++	++++	++++	++++	++	0	0	0	0	0	0
Brain	++++	++++	++++	++++	++++	0	0	0	0	0	0	0
Kidney, double quantity	++++	++++	++++	++++	++++	+++	+	0	0	0	0	0
Liver, double quantity	++++	++++	++++	++++	++++	++	0	0	0	0	0	0
Brain, double quantity	++++	++++	++++	++++	++++	0	0	0	0	0	0	0
Kidney, liver	++	0	0	0	0	0	0	0	0	0	0	0
Kidney, brain	+++	0	0	0	0	0	0	0	0	0	0	0
Liver, brain	+++	+	0	0	0	0	0	0	0	0	0	0

Protocol 10. Brain serum; brain antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS									
	0.02	0.01	0.009	0.008	0.006	0.005	0.004	0.003	0.002	0.001
	++++	++++	++++	++++	++++	++++	++++	++++	+++	0
Kidney	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Liver	++++	++++	++++	++++	++++	++++	++++	++++	+++	0
Brain	++++	+++	++	+	0	0	0	0	0	0
Kidney, double quantity	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Liver, double quantity	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Brain, double quantity	++++	+++	+	0	0	0	0	0	0	0
Kidney, liver	++++	++++	++++	++++	+++	+	0	0	0	0
Kidney, brain	++++	+++	0	0	0	0	0	0	0	0
Liver, brain	++++	++	0	0	0	0	0	0	0	0

Protocol 11. Antibrain serum; corpuscle antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS												
	0.05	0.03	0.02	0.01	0.009	0.008	0.006	0.005	0.004	0.003	0.002	0.001	0.0005
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0
Kidney	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0	0	0
Liver	++++	++++	++++	++++	++++	++++	++++	++	+	0	0	0	0
Brain	++++	++++	++++	++++	0	0	0	0	0	0	0	0	0
Kidney, double quantity	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	0	0	0
Liver, double quantity	++++	++++	++++	++++	++++	++++	++++	0	0	0	0	0	0
Brain, double quantity	++++	++++	++++	++++	++	0	0	0	0	0	0	0	0
Kidney, liver	++++	++++	++++	++++	++++	+	0	0	0	0	0	0	0
Kidney, brain	++++	0	0	0	0	0	0	0	0	0	0	0	0
Liver, brain	++++	+++	0	0	0	0	0	0	0	0	0	0	0

Protocol 12. Antibrain serum; corpuscle antigen

ABSORBENTS	QUANTITY OF SERUM IN CUBIC CENTIMETERS						
	0.1	0.08	0.06	0.05	0.03	0.01	0.08
	++++	++++	++++	++++	++++	++	0
Kidney	++++	++++	++++	++++	++	0	0
Liver	++++	++++	++++	++++	++	0	0
Brain	++++	++++	++++	++	0	0	0
Brain, double quantity	++++	++++	++++	++	0	0	0
Kidney, liver	++	0	0	0	0	0	0
Kidney, brain	++++	+++	++	0	0	0	0
Liver, brain	++++	+++	++	0	0	0	0

of antibodies, some markedly specific and limited in their reactive range, others probably more loosely specific and having a wide range of action.

In the following protocols are given examples of the various experiments; in all about fifty similar experiments were carried out. We show here the results of the complement fixation reactions with various sera tested against various antigens, both before and after being absorbed with various tissue emulsions.

We have omitted from the protocols the data concerning the hemolytic system used and the amount of antigen (tissue) used as these were determined by preliminary titration before each day's experiments were actually begun.

CONCLUSIONS

1. There exist apparently, in the various tissues, substances or chemical compounds which are more definitely related to and show a specificity to certain substances contained in other tissues. It is, however, not certain that these non-homologous tissue specific substances are chemically and immunologically identical with the tissue specific substance of the particular tissues. They may only be similar.

2. The demonstration of this type of antigen in the tissue and of the corresponding antibody in the antiserum adds only to the evidence of the complexity of these tissue antigens and antibodies. Using, as we have done, the entire organ it would naturally be expected that we should find the interpretation of our results complicated by this fact, but it has apparently been possible to disassociate and analyse the various immunological relations of the components of these complex antigens.

REFERENCES

(1) Fleisher, Hall and Arnstein: Journ. Immunology, 1920, **5**, 437.
(2) Fleisher and Arnstein: Journ. Immunology, 1921, **6**, 223.

BACILLUS DIPHTHERIAE[1]

IMMUNOLOGICAL TYPES; TOXIN-ANTITOXIN RELATIONSHIP

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Recently it has been demonstrated that at least two immunological groups exist among virulent diphtheria bacilli. This discovery immediately raised the question of the identity of the toxins produced by each group, and their reactions to standard antitoxin produced by the injection of toxin derived from a single strain. The question of the identity of the toxins produced by the two groups is important not only from the standpoint of scientific interest, but also from the standpoint of the production of a proper antitoxin suitable for therapeutic use in all cases.

Havens (1) described two distinct biologic groups of the *Bacillus diphtheriae*. In studying 206 different strains he found that 82 per cent of them were included in one group and the remaining 18 per cent composed a second group, as evidenced by the agglutination reaction. Havens also prepared toxins from the organisms of both groups. On testing these toxins against standard antitoxin (prepared by group I organisms) he came to the conclusion that a difference existed between the two toxins. The neutralization of group I toxin was complete, of group II partial or incomplete. Such differences as existed were not as sharply defined as that shown by the agglutination reaction with the organisms themselves.

[1] Read before the Eastern Pennsylvania Chapter Society American Bacteriologists, November 8, 1921.

At the meeting of the "American Association of Pathologists and Bacteriologists," held in Cleveland, March, 1921, Dr. W. H. Park reported observations similar to ours, in that group II toxin is neutralized by antitoxin obtained by immunization with toxin from group I organisms.

Inasmuch as diphtheria antitoxin is universally prepared only from strains that fall in the first and largest group, the following studies were undertaken in an attempt to corroborate Havens' results.

In these experiments, seven strains of *B. diphtheriae*, obtained from the following sources, were used:

Strain P8	Dr. W. H. Park, New York	Unclassified
Strain 1058	Dr. J. W. Bunker, Detroit, Mich	Unclassified
Strain 1287	Dr. L. C. Havens, Baltimore, Md	Classed as group I
Strain 1288	Dr. L. C. Havens, Baltimore, Md	Classed as group II
Strain 473	Dr. Anna Williams, New York	Unclassified
Strain KL4, Strain KL6	Dr. G. H. Robinson, Baltimore, Md	Classed as group II

I. IMMUNOLOGICAL GROUPING

Agglutinating serums were prepared from strain 1058, suspected to be group I, and from strain KL4, group II.

Twenty-hour cultures on Loeffler's blood serum were used. The growth was removed with a loop, suspended in sterile physiological salt solution and heated at 60°C. for twenty minutes. Rabbits were given the following injections of this antigen. At three-day intervals:

500 million organisms subcutaneously
1000 million organisms subcutaneously
2000 million organisms subcutaneously
1500 million organisms subcutaneously
3000 million organisms subcutaneously
3000 million organisms subcutaneously

Then at daily intervals:

2000 million organisms intravenously
2000 million organisms intravenously
2000 million organisms intravenously
6000 million organisms intravenously

After an interval of seven days from the last injection the animals were bled to death and the sera collected. All the cultures were than tested against these two sera by means of the agglutination reaction.

Agglutination technic

The antigen for the agglutination test was made from twenty-hour cultures on Loeffler's blood serum, the growth being suspended in salt solution. After heating the suspension in a water bath at 60°C. for twenty minutes, it was centrifuged and the supernatant fluid discarded. The concentrated antigen was then diluted until one cubic centimeter contained 2000 million organisms. Thus the final suspension of the antigen in the test contained 1000 million organisms per cubic centimeter. The tubes were incubated in a water-bath for two hours at 37.5°C., and then placed in the refrigerator and read the next morning.

The results of this test reveal a marked difference between the two sera in their reaction on the antigens. Serum prepared from strain 1058 agglutinated four of the six strains tested in dilution of 1:640; including strains 1287 and Park and Williams 8, which were classified by Havens as group I. Park and Williams 8 strain is universally employed in the production of diphtheria toxin. Serum obtained from strain KL4, agglutinated only the two group II strains in dilution of 1:640. In both sera agglutinins common for all the strains were present in dilutions of 1:10 to 1:80; nevertheless, two groups are distinctly evident among these strains.

After the cultures were grouped by the agglutination reaction as outlined in the preceding table, toxins were prepared from two strains of each group and tested against group I antitoxin.

II. TOXIN-ANTITOXIN RELATIONSHIP

The bouillon employed for the preparation of toxin was made from fresh bob-veal. (The meat is allowed to ferment overnight with *B. coli* and is then boiled and 1 per cent Wittes peptone and 0.5 per cent sodium chloride are added, to the strained meat infusion.) The final reaction of the bouillon ranged between pH 7.5 and pH 7.7. For the production of the experimental toxins, two Erlenmeyer flasks, each containing 350 cc. of bouillon, were used for each strain. The strains employed in making group I toxins were Park 8 and Havens 1287. The group II

TABLE 1

Culture	Serum Group I (1058)									Serum Group II (KL4)									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Control
P8	++++	++++	++++	++++	++++	++++	+++	+	−	+++	+++	+++	++	+	−	−	−	−	−
1058	++++	++++	++++	++++	++++	++++	+++	+	−	+++	+++	++	+	−	−	−	−	−	−
1287	++++	++++	++++	++++	++++	++++	+++	−	−	+++	+++	+++	+++	+	−	−	−	−	−
473	++++	++++	++++	++++	++++	++++	+++	−	−	+++	+++	+++	+	−	−	−	−	−	−
KL4	+++	++	+	−	−	−	−	−	−	++++	++++	++++	++++	++++	++++	+++	+	−	−
KL6	+++	+++	++	+	−	−	−	−	−	++++	++++	++++	++++	++++	++++	+++	+	−	−

toxins were made from strains KL4 and KL6. The toxin was considered ripe on the seventh day of incubation at 36°C. At this point the growth was shaken down, 0.5 per cent phenol added, and the flasks allowed to stand at room temperature for twenty-four to thirty-six hours, before filtering. After filtering through Mandler filters these toxins were tested for potency in the usual way on 250-gram guinea-pigs. The following readings made on the fourth day, show the number of minimal lethal doses per cubic centimeter in each lot.

TABLE 2

Potency tests of toxin

TOXIN NUMBER	CULTURE	GROUP	MINIMAL LETHAL DOSE	MINIMAL LETHAL DOSE PER CUBIC CENTIMETER
110	P8	1	0.0025	400
110A	KL4	2	0.025	40
110B	KL6	2	0.02	50
110C	1287	1	0.002	500
111	P8	1	0.005	200
111A	KL4	2	0.0133	75
111B	KL6	2	0.0133	75
111C	1287	1	0.00285	350
112	P8	1	0.00333	300
112A	KL4	2	0.01	100
112B	KL6	2	0.01	100
112C	1287	1	0.00333	300
113	P8	1	0.0025	400
113A	KL4	2	0.0133	75
113B	KL6	2	0.0133	75
113C	1287	1	0.0025	400

From the foregoing table it will be noted that group I strains yielded toxin testing 300 to 500 minimal lethal doses per cubic centimeter while the potency of group II toxin was only 40 to 100 minimal lethal doses per cubic centimeter, although both groups were grown in the same medium. For the difference in this respect we cannot account.

The neutralizing relationship of these toxins to antitoxin was determined by injecting healthy guinea-pigs, weighing 250 grams, with varying amounts of toxin plus one immunity unit of antitoxin, of the standard of the United States Hygienic

Laboratory (2). We consider this phase of the investigation to be of prime importance since the results of the tests, as given in tables 3 and 4, show the neutralizing power of standard antitoxin to be equally effective against group I and group II toxins.

TABLE 3

Protection tests

TOXIN NUMBER	GROUP	NUMBER OF MINIMAL LETHAL DOSES INJECTED	UNITS ANTITOXIN	ONE DAY	TWO DAYS	THREE DAYS	FOUR DAYS
110-A	2	10	1	L	L	L	L
		40	1	L	L	L	L
		80	1	L	L	L	L
110-B	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	L	D	
110-C	1	50	1	L	L	L	L
		100	1	L	D		
		200	1	D			
111	1	10	1	L	L	L	L
		50	1	L	D		
		100	1	D			
111-A	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	L	D	
111-B	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		
111-C	1	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		

L = lived; D = dead.

The amount of protection in one immunity unit of standard antitoxin against the toxins of either group, was found to range between 50 and 100 minimal lethal doses with the exception of two lots of group I toxin (lots 111 and 112), in which the protection fell below 50 minimal lethal doses. Lot 113-A shows

proof of protection against 100 minimal lethal doses of group II toxin. This result is, we believe, due to the estimation of too high a potency for lot 113-A, so that the doses given in this

TABLE 4

Protection tests

TOXIN NUMBER	GROUP	NUMBER OF MINIMAL LETHAL DOSES INJECTED	UNITS ANTITOXIN	ONE DAY	TWO DAYS	THREE DAYS	FOUR DAYS
112	1	10	1	L	L	L	L
		50	1	L	D		
		100	1	D			
112-A	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		
112-B	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		
112-C	1	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		
113	1	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	D		
113-A	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	L	L	L
113-B	2	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	L	D	
113-C	1	10	1	L	L	L	L
		50	1	L	L	L	L
		100	1	L	L	D	

case did not contain the required amount of toxin. Every pig that died was autopsied and found to have lesions characteristic of diphtheria toxin. Those which survived showed no ill effects.

It appears from these results that one immunity unit of standard antitoxin contains enough neutralizing power to protect a guinea-pig weighing 250 grams, against 50 or more minimal lethal doses

TABLE 5

Controls for potency test

TOXIN NUMBER	GROUP	NUMBER OF MINIMAL LETHAL DOSES INJECTED	DILUTION 1:5 NORMAL HORSE SERUM	ONE DAY	TWO DAYS	THREE DAYS
112	1	10	Horse no. 4985, 1 cc.	D		
		50	Horse no. 4985, 1 cc.	D		
		100	Horse no. 4985, 1 cc.	D		
112-A	2	10	Horse no. 4985, 1 cc.	L	D	
		50	Horse no. 4985, 1 cc.	D		
		100	Horse no. 4985, 1 cc.	D		
112-B	2	10	Horse no. 4985, 1 cc.	L	D	
		50	Horse no. 4985, 1 cc.	D		
		100	Horse no. 4985, 1 cc.	D		
112-C	1	10	Horse no. 4985, 1 cc.	L	D	
		50	Horse no. 4985, 1 cc.	D		
		100	Horse no. 4985, 1 cc.	D		
113	1	10	Horse no. 4957, 1 cc.	D		
		50	Horse no. 4957, 1 cc.	D		
		100	Horse no. 4957, 1 cc.	D		
113-A	2	10	Horse no. 4957, 1 cc.	L	L	D
		50	Horse no. 4957, 1 cc.	L	D	
		100	Horse no. 4957, 1 cc.	D		
113-B	2	10	Horse no. 4957, 1 cc.	L	D	
		50	Horse no. 4957, 1 cc.	D		
		100	Horse no. 4957, 1 cc.	D		
113-C	1	10	Horse no. 4957, 1 cc.	L	L	D
		50	Horse no. 4957, 1 cc.	L	D	
		100	Horse no. 4957, 1 cc.	D		

of toxin of either group. The protection seems to be just as effective against group II toxin as against group I toxin.

It was deemed desirable to control the protection tests with normal horse serum. Sera from two horses diluted in the same

proportion as standard antitoxin, were used against several lots of toxin to ascertain whether or not horse serum in such dilutions would protect guinea-pigs against large doses of diphtheria toxin.

From these control tests we are furnished with enough evidence to show that normal horse serum does not possess any protection for guinea-pigs against group I or group II toxin, when used in the same proportion as standard antitoxin.

In view of the fact that protection against the toxins of both groups proved to be very effective by standard antitoxin, the question of protection against virulent cultures representing both groups became important. In combating diphtheria infection, both organisms and toxin must be taken in consideration. It was therefore deemed essential to know whether antitoxin in reasonable amounts would protect guinea-pigs against virulent cultures of the *B. diphtheriae.*

The standard used in all our protective tests was one immunity unit of antitoxin, of the standard of the United States Hygienic Laboratory.

Twenty-four hour cultures on Loeffler's blood serum were washed off with sterile saline solution and diluted to contain approximately 2000 million organisms per cubic centimeter. One cubic centimeter of such dilutions was found to be fatal for guinea-pigs in forty-eight hours. The suspensions were injected subcutaneously into 250-gram guinea-pigs, immediately followed by a subcutaneous injection of one unit of standard antitoxin. The results of this experiment are as shown in table 6.

The evidence obtained in this test again emphasizes the protective value of standard antitoxin. One unit of antitoxin was found to protect against a dose of group I culture that was fatal to guinea-pigs in forty-eight hours. Therefore, only one unit of antitoxin was used against the group I strains. Against group II strains, 1, 1½ and 2 units of the antitoxin were used. One unit proved to be sufficient to protect the pigs against all but one group II strain (1288). In this case the pig died on the fourth day. However, a good protection was obtained against this strain with 1½ and 2 units of antitoxin. There seems to be

some difference between strain 1288 and the other two strains of the homologous group as far as toxicity is concerned. In those cases where 1½ and 2 units of antitoxin were used a much higher degree of protection was obtained than with one unit. There was less edema and necrosis and very little loss of weight.

TABLE 6

Virulent culture protection tests

CULTURE	GROUP	AMOUNT OF CULTURE (2000 MILLION PER CUBIC CENTIMETER)	UNITS ANTITOXIN	TWO DAYS	THREE DAYS	FOUR DAYS	FIVE DAYS	SIX DAYS	SEVEN DAYS
1058	I	1	1	L	L	L	L	L	L, normal
1058	I	1	None	Dead					
473	I	1	1	L	L	L	L	L	L, lost weight
473	I	1	None	Dead					
1288	II	1	1	L	L	Dead			
1288	II	1	1½	L	L	L	L	L	L, normal
1288	II	1	2	L	L	L	L	L	L, normal
1288	II	1	None	Dead					
1288	II	1	None	Dead					
KL4	II	1	1	L	L	L	L	L	L, lost weight
KL4	II	1	1½	L	L	L	L	L	L, normal
KL4	II	1	2	L	L	L	L	L	L, normal
KL4	II	1	None	Dead					
KL4	II	1	None	Dead					
KL6	II	1	1	L	L	L	L	L	L, lost weight
KL6	II	1	1½	L	L	L	L	L	L, lost weight
KL6	II	1	2	L	L	L	L	L	L, normal
KL6	II	1	None	Dead					
KL6	II	1	None	Dead					

L = lived.

All the control pigs when autopsied revealed lesions characteristic of diphtheria poisoning.

These tests were repeated with identical results. Washed cultures were also used, but no difference in virulence could be noted between washed and unwashed cultures.

SUMMARY

At least two distinct biologic groups of *B. diphtheriae* exist among virulent strains, as differentiated by the agglutination reaction.

We were unable to confirm the contention, that group II toxin is not neutralized by standard antitoxin to the same extent as group I toxin.

The results of the experiments presented, lead us to the conclusion that diphtheria antitoxin as produced by the injection of toxin obtained from group I strains, neutralized equally well the toxins produced by either group I or group II organisms.

One, one and a half, and two units of standard antitoxin injected simultaneously with large doses of virulent cultures, protect guinea-pigs against both types of *B. diphtheriae*.

We wish to acknowledge the courtesy of Drs. R. D. Meader, G. H. Robinson, and L. C. Havens, in supplying us with group II cultures.

REFERENCES

(1) Havens, Leon C.: Biologic studies of diphtheria bacillus. Jour. of Inf. Diseases, 1920, 26, no. 5, p. 388.
(2) Rosenau, M. J.: Hyg. Lab. Bulletin, no. 21, 1905.

BRONCHIAL ASTHMA AND ALLIED CONDITIONS

CLINICAL AND IMMUNOLOGICAL OBSERVATIONS

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An asthma clinic was established in the out-patient department of Bellevue Hospital, New York City, in October, 1919. For the year 1919–1920, the scope of the work included a detailed study of the clinical aspects of asthma, which was published (1). During the year 1920–1921 the following investigations were attempted, the results of which are embraced in this paper: (*a*) A continued study of the clinical considerations of asthma, (*b*) observations on skin reactions, (*c*) effects of vaccine therapy.

During the year, 105 cases of bronchial asthma were studied in detail; 25 of pollen sensitization; 2 of urticaria, and 2 of angioneurotic edema.

CLINICAL OBSERVATIONS

From a practical standpoint, it is important that cases of bronchial asthma be recognized clinically as speedily as possible, since special methods for diagnosis and relief of the condition are attempted. The urgency of the presenting symptom, however, leads to quick transfer to the asthma clinic, when the dyspnea is supposedly asthmatic. The first task of the clinic, therefore, is to eliminate those cases which do not belong to it.

Apparently, the most frequent diseases superficially mistaken for bronchial asthma in this clinic are: Chronic cardiac disease, chronic nephritis, acute bronchitis, chronic pulmonary tuberculosis; much less frequently subjective dyspnea in "nervous" subjects, hysterical tachypnea, obesity, and early lobar pneu-

monia in advanced age. All of these patients have complained of "wheezing" and "choking," and some presented chest signs not unlike bronchial asthma. Through lack of careful history and physical examination, these patients were referred to the asthma clinic.

After such elimination, there is left a large group of cases complaining of difficult respiration (usually described as "wheezing," "choking," or "tight breathing"), which comes on periodically, and especially at night. In some cases, particular stress is laid on cough and expectoration, which may be entirely absent in other cases. It is well known that the characteristic physical signs of bronchial asthma, chronic bronchitis, pulmonary emphysema, may or may not be elicited.

It is in these patients that special means of differentiation are followed. We have attempted to divide them under the following headings:

1. Bronchial asthma; simple, uncomplicated.
2. Chronic bronchial infection with its complications, simulating bronchial asthma.
3. Bronchial asthma, complicated by chronic bronchitis.

Possibly a fourth type, as yet unrecognized, may be chronic bronchitis complicated by bronchial asthma, and not merely simulating it—including those who have acquired sensitization to bacterial proteins.

The first type is well defined. It is characterized by a frequent family history of bronchial asthma, hay fever, or other allergic conditions; early age of onset; past history of hay fever, urticaria, eczema, or angioneurotic edema. The paroxysms are sudden in onset, and are usually *followed* by cough, with production of mucoid sputum. Physical examination often reveals the constitutional defect of status lymphaticus or its borderline conditions. Likewise, the blood pressure is characteristically low, and there is often increased sensitiveness to epinephrin. There is absence of evidences of bronchitis, and relative infrequency of emphysema, as shown by physical examination and x-ray. This group shows a high percentage of skin reactions to foreign proteins.

Eosinophilia in the blood is present in this type of simple bronchial asthma. The sputum, on gross examination, as seen in Petri plates against a black background, is homogeneous, viscid, and transparent, excepting the small hazy coiled streaks known as "perles." Large masses of eosinophiles are present, as many as 50 per oil immersion field. Polymorphonuclear neutrophiles are relatively few.

The characteristics of the second type (bronchial infection simulating bronchial asthma) is in contrast with that of the first. A late age of onset is the rule, with history of cough, and profuse expectoration of long standing, preceding the asthmatic symptom. They do not give histories of allergic conditions, as mentioned in the above group, or family histories of these ailments. They usually state that their attacks "begin with a cold." Cough and expectoration are always prominent, and *precede* the attacks, which are not sudden, without warning, as in the first type. Physical examination usually shows the signs produced by exudate in the bronchioles and bronchi, whether the patient is suffering from asthmatic dyspnea or not. Pulmonary emphysema is frequent. In contrast to the first type, in which constitutional defects were found to be common, the physique is usually robust. Blood pressures are not characteristically low, and the response to epinephrin is normal. They do not, as a class, show sensitiveness to foreign protein.

The sputum in this second type is mucopurulent, opaque, without "perles." There are many polymorphonuclear neutrophile cells, and only occasional eosinophiles.

The third type is primarily type 1, on which infection is superimposed. It is differentiated from the second type by the history, physical examination, and sputum, all of which retain characteristics of the first type. The sputum is mucoid, containing varying amounts of mucopurulent material. "Perles," when seen, are opaque. Polymorphonuclear neutrophile cells are prominent in proportion as the infection dominates the original condition.

IMMUNOLOGICAL REACTIONS

During the year, observations were made on the "skin reaction" as a diagnostic aid in asthma and allied conditions. The mechanism of the reaction has not been established. That the skin reaction sometimes demonstrates the exciting cause of symptoms, is a well known fact. Since often no amount of questioning reveals the cause, and as the removal of it gives immediate relief, there is justification for continued study of this diagnostic aid.

A certain proportion of individuals suffering with asthma or hay fever react with the formation of an urticarial wheal when a small amount of the substance which can originate an attack is brought in contact with a scratch, or injected into the skin. The wheal exhibits irregular edges and pseudopodia develop within twenty minutes, and are usually surrounded by an area of erythema, accompanied sometimes by swelling and other signs of inflammation. The site itches and occasionally remains red and swollen for several days. Regardless of method employed, a marked reaction in a definitely sensitive skin never is difficult to elicit.

Comparison of methods

More tests can be made with less trouble, and time, at the same sitting, with the scratch method. The resulting mark, which, in unusual cases, may last several months, is more pronounced with the scratch than with the intracutaneous method. With dry preparations there is no way in which to tell when they have lost their potency. With solutions, contaminations which destroy the potency can be readily seen. Also, when a precipitate forms in a solution, the potency may be decreasing. One such solution which had given us very good reactions for several months, developed a precipitate. Cultures proved it to be sterile. Upon testing, negative reactions were obtained in some skins which then reacted well to a fresh solution.

For an out patient dispensary where expense had to be considered, an inexpensive syringe was devised, and has given satisfactory service during the past six months. This consists of a

simple capillary tube of "non-sol" glass, with a flange at the wide end, over which a rubber bulb, with a thick wall, and one large perforation, is fitted.

Clinical results showed that the slight tenchical advantages of the scratch method were outweighed by the increased reliability of the intracutaneous method. To illustrate with a case:

A. B. developed asthma only when in contact with horses. A horse could pass, or he could ride a horse for a few minutes without symptoms, but after close contact for four or five minutes, he invariably began to wheeze. On May 1, 1921, he was tested intracutaneously on the right forearm with a solution of horse dander prepared by Dr. Coca's method. Two inches away, on the same arm, a dry commercial preparation was applied by means of a $\frac{1}{8}$-inch superficial scratch, upon which a drop of $\frac{N}{10}$ NaOH was applied, and then dry powder stirred into it. A control injection of horse serum was made. After twenty minutes, the in-

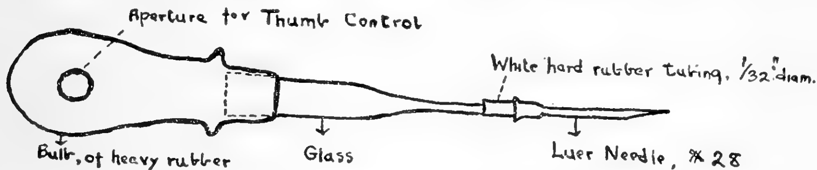

Fig. 1. Syringe for Intracutaneous Injection

tracutaneous reactions showed an irregular wheal, 3 by 2.5 cm., surrounded by erythema. The patient complained of itching at the site of injection. There was no reaction at the site of the scratch. The solution of horse dander, which gave this reaction, was injected into ten other asthma patients with negative results. The horse serum gave no reaction. Similarly it was found that in fifteen instances (table 1) there was no definite response to the commercial preparations put on by the scratch method, although history and intracutaneous tests were positive. The ragweed and timothy scratch reactions performed with commercial solutions proved to be potent on the more sentitive skins. The dog hair, cat hair, chicken feather, and rabbit hair dry preparations had been received within three months and were then kept dry in the laboratory. The commercial pollen extracts gave a good reaction in most cases. The dry products, however, although effective in most cases, were so uncertain that they were unsatisfactory for routine use. Positive reactions were designated as +, doubtful as ± and negative as −.

It is not possible to use the calibration devised by Walker in comparing these two methods. One can make no definite ruling as to the reading of reactions. Some reactions up to 1 cm. in diameter were considered negative. The normal varies with different skins. The normal on an irritable skin cannot be compared with the normal on an unresponsive skin. It emphasizes the fact that questionably positive reactions must be measured

TABLE 1

Comparison of reactions in sensitive individuals. Commercial preparations used with the scratch method and Dr. Coca's solutions intracutaneously

PATIENT	PROTEIN	HISTORY OF CONTACT	COCA SOLUTION INTRA-CUTANEOUSLY	COMMERCIAL PREPARATION, SCRATCH METHOD
D.	Chicken feathers	+	+	−
I. D.	Dog hair	+	+	−
S.	Cat hair	+	+	+
B.	Horse dander	+	+	−
M. C.	Horse dander	?	+	−
M. C.	Rabbit hair	?	+	−
M. C.	Chicken feathers	+	+	−
I. D.	Horse dander	+	+	−
V. E.	Chicken feathers	+	+	−
H. B.	Ragweed	+	+	±
O. H.	Timothy	−	+	−
O. H.	Ragweed	+	+	−
S. R.	Ragweed	+	+	−
E. R.	Ragweed	+	+	−
B. B.	Ragweed	+	+	−
H.	Ragweed	+	+	−

Although, in seventeen other instances, in sensitive individuals, both scratch and intracutaneous methods were positive, in no case was the scratch positive and the intracutaneous negative.

parallel with control reactions on the same individual. These are the borderline reactions; they occur with the scratch as well as with the intracutaneous method. In such cases, an opinion can be arrived at only by repeated tests on different days, and with careful investigation of possible contact. Another well-known fact which has at times led to misinterpretation, is a positive reaction in a clinically non-sensitive individual. One such positive reaction will not necessarily establish a diagnosis.

Comparison of the two methods with the same solutions

To compare the scratch and intracutaneous methods on the same individual with the same solutions, the following experiment was performed: Ten patients known to be sensitive to ragweed pollen were injected on the anterior surface of the forearm, using

TABLE 2

Comparison of strengths of reactions to cutaneous and intracutaneous applications of ragweed solutions of varying pollen content, in ten sensitive individuals

SOLUTION	POSITIVE	DOUBTFUL	NEGATIVE
Solution A:			
Intracutaneous	8	1	1
Scratch	5	1	4
Solution B:			
Intracutaneous	10	0	0
Scratch	4	0	6
Solution C:			
Intracutaneous	6	2	2
Scratch	1	1	8
Solution D:			
Intracutaneous	5	2	3
Scratch	1	0	9
Control Patients:			
Solution B:			
Intracutaneous	0	0	2
Scratch	0	0	2

Solution A: Ragweed pollen solution 0.3 mgm. N per cubic centimeter.
Solution B: Ragweed pollen solution 1:100.
Solution C: Ragweed pollen solution 1:1000.
Solution D: Ragweed pollen solution 1:5000.

four solutions of different strengths of ragweed pollen. Solution A, ragweed solution with 0.3 per thousand nitrogen content. The other three were freshly made solutions. (Coca method). Solution B, 1:100; Solution C, 1:1000; Solution D, 1:5000. Weight of pollen in grams was the factor used in determining the strength of dilutions. The middle anterior one-third of the forearm was used, alternately placing the strongest solution superiorly and

inferiorly on the different patients. Two inches away, and parallel with the injections, scratches were made about $\frac{1}{8}$ inch long, and deep enough so that small red points were just visible. These scratches were kept covered with the various solutions for twenty minutes. At the end of that time, readings were made by measuring the longest and broadest diameter in millimeters. The results are tabulated in table 2. The two strongest solutions were injected at the same time into two normal individuals, and had been used on many other patients without reactions. Control solutions were also used on all these sensitive cases with negative results.

It is therefore evident that certain individuals, whose asthma or hay fever is brought on by specific proteins, may give a skin reaction to a solution of that protein when it is injected intracutaneously, and show no reaction when the same solution is applied by means of a scratch. Walker (2) also found this to hold true.

Relation of size of scratch to size of reaction

To determine whether the size of the scratch influences the size of the reaction, the following experiment was performed: As in the above experiment, the middle anterior third of the forearm was used. Eleven patients whose skins were definitely sensitive to ragweed were chosen. Two or more scratches of lengths varying from 1 to 11.5 mm., and just deep enough to draw blood, were made. The scratches were covered with decinormal NaOH and dry ragweed pollen mixed with the fluid. The reactions were read in twenty minutes. The results are recorded in table 3. The size of reaction in breadth varies with the length of the cut, but not directly, due perhaps to the irregular distribution of lymph spaces and channels.

Influence of location on reaction

Schloss (3) believes that the size of reaction varies, depending upon the location on the arm. To test this statement, fourteen sensitive patients were given intracutaneous injections of ragweed pollen solution at the elbow fold anteriorly, and a second

injection of this solution, of like amount, was given 10 cm. below. The reactions were read in twenty minutes. Results are shown in figure 2. The reaction at the elbow was never smaller, and was usually larger than the reaction at the region nearer the wrist. There was always more erythema at the elbow site.

TABLE 3

Relation of length of scratch to maximum diameter of reaction measured in millimeters

PATIENT	LENGTH OF SCRATCH	WIDTH OF REACTION
N. E.	11.5	11.0
R. M.	10.5	5.0
M. M.	9.5	13.0
B. X.	8.5	4.0
N.	8.0	15.0
R.	8.0	13.0
B.	8.0	11.0
F.	8.0	8.0
R. O.	7.5	5.5
R. U.	7.0	7.0
R.	4.5	11.0
N.	4.0	9.0
R.	3.0	17.0
M. M.	3.0	11.0
R.	3.0	9.0
N.	3.0	8.0
N.	3.0	7.0
N. E.	2.5	7.5
B.	2.5	7.0
F.	2.5	3.0
B. X.	2.5	1.0
N.	2.0	7.0
R. M.	2.0	4.5
R. U.	2.0	4.0

Other factors which influence the size of the reaction

Another factor which influences the size of reaction is the degree of sensitivity of the cells. This does not appear to be proportionate to the severity of the clinical symptoms. The reactive power of the skin is variable, since the same solution of ragweed, injected in like amounts into the skins of one normal and two

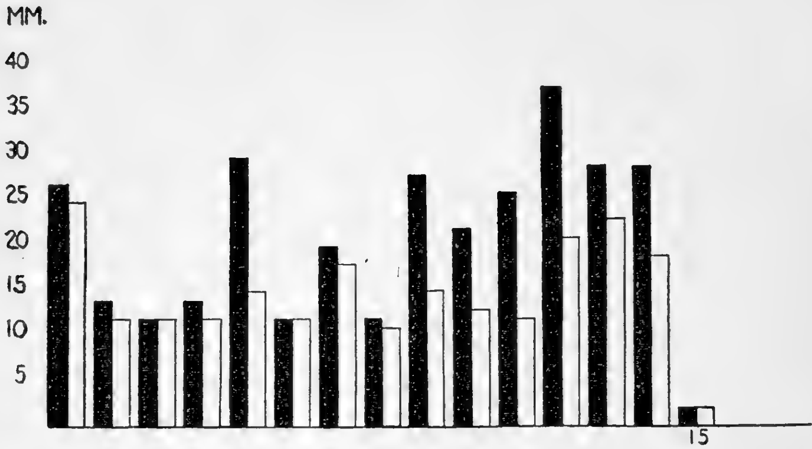

FIG. 2. DIAMETERS IN MILLIMETERS OF SKIN REACTIONS TO A SOLUTION OF RAGWEED POLLEN (0.3 MGM. N PER CUBIC CENTIMETER)

Injected into each of fourteen patients at the anterior elbow fold and 10 cm. below.

Shaded area: Diameter of reaction at elbow fold.
Clear area: Diameter of reaction 10 cm. below elbow fold.
15: Control (non-sensitive) patient.

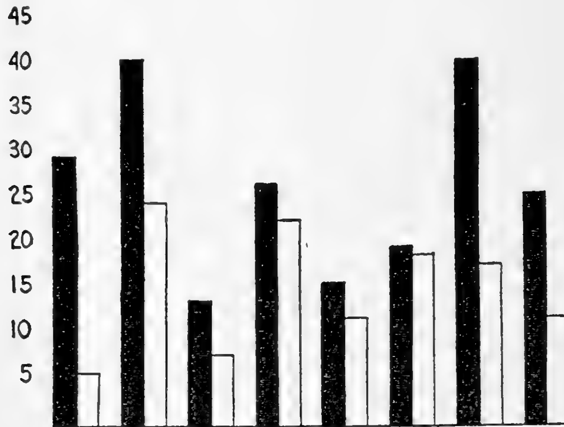

FIG. 3. DIAMETER IN MILLIMETERS OF SKIN REACTIONS TO VARYING QUANTITIES OF SOLUTIONS OF RAGWEED POLLEN (0.3 MGM. N PER CUBIC CENTIMETER) IN EIGHT PATIENTS

Shaded area: 0.04 cc. injected.
Clear area: 0.01 cc. injected.

sensitive individuals gave the following experimental results. The first showed no reaction while the two sensitive patients showed wheals. One of the wheals measured 2 cm. in diameter, the other 10 cm. by 5 cm.

Again, the strength of the protein solution brought in contact with the cells is a factor which helps to determine the size of reaction. This is shown in table 2, where the same amount of fluid was injected in each instance. Some individuals fail to respond to a dilution in which the protein proportion is less than 1:100. This fact has been made use of to determine the initial dose of curative injection.

When different quantities of protein solutions of equal strength are employed, a similar relation is noted. Eight sensitive patients were injected on the right forearm with 0.01 cc. and 0.04 cc. of a ragweed solution containing 0.3 mgm. of nitrogen per cubic centimeter. The reactions were measured at the end of twenty minutes. The results are shown in figure 3. The size of wheal is shown to increase with increase in the amount of protein solution injection, though not always in proportion with it.

Variations in skin reactions at different times

Another point noted was a variation in the skin reaction from week to week. In fourteen sensitive cases receiving identical inoculations of ragweed pollen solutions, reactions were measured for a period of several weeks. In no case did the skin reaction become negative. In eleven cases there was a definite decrease, both as to size of wheal, amount of erythema, itching, and duration of resultant swelling. The effect was more marked in cases which had at first given violent reactions. In the remaining three cases, there seemed to be no change in the response. That, after a series of prophylactic inoculations, an individual can go through a season without symptoms, and yet constantly show a positive skin reaction, is well known. Normal variations were also watched in sensitive cases not under treatment. The question whether the skin reaction shows variation in intensity, or complete disappearance at different times, is being studied.

The skin reactions were, therefore, found to be influenced by the following factors: (*a*) The preparation used; (*b*) the method of application; (*c*) the length of scratch made; (*d*) the site of injection; (*e*) the degree of cellular sensitivity; (*f*) the amount of protein brought in contact with the cells, and the amount of solution injected.

Local skin desensitization

A point still in question is local skin desensitization. Mackenzie (4) showed that by repeated injections at the same site on the same day, he could cause a disappearance of the reaction. This was repeated on two cases, as follows: The first was a male nurse sensitive to timothy pollen. He was injected nine times in the same site with timothy solution, 0.6 mgm. of nitrogen per centimeter. The first injection was given at 9:30 a.m.; the last at 6:30 p.m. The injections were approximately one hour apart. Due to the erythema and swelling which remained, it was difficult to gauge the amount injected each time. After no injection, however, did the skin fail to respond with a new wheal. After the eighth injection, the patient had symptoms of hay fever; also, two hours after the ninth. The eighth and ninth reactions were the largest, giving wheals with irregular edges and pseudopodia. The next morning, the forearm was still red and swollen, and another injection at the same site was followed by a markedly positive reaction.

The second case was a ragweed sensitive patient. Four injections were given during the course of an afternoon. After no injection did the cells fail to respond, and there was apparently no decrease in reaction.

To test out the effect of weekly injections at the same site, two patients were used. After several injections, there seemed to be a definite decrease in the reactive power of these cells. The wheal became smaller, the erythema less, and when seen on the succeeding day, the site of continuous injection was clear. The control (new site) still showed a swollen area of erythema several centimeters in diameter. One patient was sensitive to both ragweed and timothy. Both decreased as stated above, and

when, after three months the injections were reversed, the cells responded as at a new site. Injections were then discontinued for a period of three weeks. When the site was then inoculated, it responded as a fresh site. On the following day, it was still red and swollen as badly as the control site. The effect was apparently only temporary, and there was no permanent increase or decrease in the power of the local cells to react.

General reactions following intracutaneous injections

One of our patients developed symptoms of hay fever after a test injection. Another patient, a nurse who had infrequent attacks of asthma, was tested out with a great variety of proteins without result. She had noticed that, after being in the ward where the children had their hair treated with delphinium, she always felt tightness in her chest. She was injected with a very small amount of the tincture of delphinium into the skin. In ten minutes no wheal appeared. Twenty minutes later, there was a large wheal at the site of injection, and an attack of asthma occurred, worse than any heretofore. Ten minims of adrenalin relieved her. Two hours later, she suddenly broke out with intense urticaria, the wheals developing over her entire body, head and extremities, with intense itching. Again in two hours she had another attack of asthma, which was again relieved by adrenalin. She has been feeling perfectly well since, but she has been careful to avoid contact with delphinium.

Several of the patients, after the first injection, had swollen and red arms. In one instance, the swelling and redness extended to six inches above the elbow. There was no pain, and the patient continued with his work. These untoward reactions are unusual.

Vaccine therapy

Recognizing the association of evidences of bronchial infection and asthma in many cases, the effects of vaccine therapy were studied. At the outset, autogenous vaccines were made from the predominating organisms in washed bronchial sputum specimens. Most of these bacteria were Gram-positive cocci. Eleven

patients were so treated. The initial dose was approximately one hundred million, and the interval once a week. Ninety-four injections in all were given. Clinical improvement in such cases must be measured largely by the patients' statements. Eight cases were thus recorded distinctly improved; there was no change in the remaining three.

After several weeks of such treatment, many of the vaccines were lost through an accident. Temporarily, two vaccines were used, each of a virulent staphylococcus aureus strain, and it was surprising to find that apparent clinical improvement continued regardless of the nature of the organism predominant in the patient's sputum.

An effort was then made to determine whether this effect were merely one of non-specific protein, and a typhoid-paratyphoid stock vaccine mixture was next given. Although slight continued improvement was noted in six of the thirteen cases treated, two who had been doing well on the former regime, became distinctly worse. No improvement was noted in the remaining four.

After six weeks of this treatment, a stock staphylococcus vaccine was used, and seventeen cases were treated with this. Improvement was recorded in ten, questionable improvement in one, and none in the remaining six.

The best results were obtained when vaccines were given in relatively small doses (100,000,000 of staphylococcus aureus), at the outset, with an increment never larger than the original dose. Two interesting observations were made: This dose would frequently cause improvement for but three or four days, and was more effective when given twice a week. Likewise, when the dosage was increased too rapidly, relapse would frequently occur. In some cases where improvement was not recorded, the asthmatic paroxysms disappeared, but the cough became worse.

The difference in effect of the various types of vaccine is not impressive, excepting that in no instance when typhoid vaccine was used, did the degree of improvement approximate that of the other organisms employed. Too few cases have been treated, to draw conclusions, but the inference is made, that, in asthma associated with signs of bronchial infection, vaccines, if given

cautiously, are worth a trial. In some instances where other measures availed little, improvement began almost immediately after vaccine therapy was instituted. In others, the results were wholly disappointing. How these agents act, has not been determined, and further study is planned.

REFERENCES

(1) Alexander, H. L., and Paddock, R.: Arch. Int. Med., 1921, **27**, 184.
(2) Walker, I. C.: Jour. Med. Research, 1917, **37**, 412.
(3) Schloss, O. M.: Am. Jour. Dis. Child., 1920, **19**, 433.
(4) Mackenzie, G. M.: Proc. Soc. Exp. Biol. and Med., 1921, **18**, 214.

STUDIES IN SPECIFIC HYPERSENSITIVENESS[1]

I. THE DIAGNOSTIC CUTANEOUS REACTION IN ALLERGY. COMPARISON OF THE INTRADERMAL METHOD (COOKE) AND THE SCRATCH METHOD (SCHLOSS)

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The first recorded experimental observation of cutaneous sensitiveness in allergy, is found in the classic monograph of Blackley (1) in which the causal relationship of the pollens to seasonal hay fever was demonstrated.

Blackley, on several occasions applied some pollen to the surface of the skin, which had been abraded as for small pox vaccination and each time, he obtained a deep and extensive swelling of the skin.

In a case of hypersensitiveness to buckwheat, reported by H. L. Smith (11) in 1909, and supposed by that author to be a case of "fagopyrismus," an experiment similar to that of Blackley was carried out by Rufus Cole. Cole used a sterilized infusion of buckwheat which he rubbed into a scarified area of the skin. This treatment resulted in the formation of a wheal of the size of a silver half-dollar and in the development of severe constitutional symptoms.

[1] This series of studies has been carried on in connection with the Clinic of Applied Immunology opened February, 1919, at the New York Hospital. During the first two years funds for the maintainance of the clinic and of the laboratory in conjunction with the clinic were supplied by private donations.

[2] Publication of this paper has been deferred in order that it might appear with the other papers of this series.—(Editors)

In the following year, Schmidt (10) reported a series of observations on the reactivity of the skin to Puro,[3] and a trypsin digested milk. The purpose of Schmidt's investigation was to search for any possible differences in the cutaneous reaction to these materials. In this main idea he failed, probably because no suitable cases of hypersensitiveness to the substances in his preparations was encountered.

Schmidt adopted the von Pirquet technic. The reactions that he did observe reached their greatest intensity after sixteen to eighteen hours; they were thus quite different from those elicited in allergy, which, with the von Pirquet technic are at their height within thirty minutes.

After Cole's experiment, the first successful attempt to make diagnostic use of the skin reactivity in allergy was reported by Oscar M. Schloss (8) in 1912 in a case of allergy to egg, almond and oat. Schloss used the von Pirquet borer for applying the tests, which were made with isolated proteins of eggwhite and preparations from the other two foods.

There is no doubt that this well conceived pioneer work of Schloss inspired much of the subsequent investigation of others, yet acknowledgement of his service is omitted in most of the literature of the subject.

Goodale (6), in 1916 wrote, "It has been known for some years that an individual sensitized to a given proteid may exhibit a characteristic reaction if the proteid is brought in a soluble form in sufficient concentration into contact with a scratch in the skin."

I. Chandler Walker (13) in 1917 wrote, "It has been known for some years that an individual sensitized to a given proteid may exhibit a characteristic reaction if the proteid is brought in a soluble form in sufficient concentration into contact with a scratch in the skin."

In all of Walker's investigations, the skin tests have been made according to the Schloss technic, and since 1916, the test

[3] A commercial preparation composed of meat extract, egg white, glycerin and salts.

material has usually been applied in the form of a powder representing the dry residue of an extract of the original material.

The method of preparation of the test substance is found in a paper by Wodehouse (17). The material is extracted with water and the extract is dried with the use of an electric fan until it becomes of a syrupy consistency. To this fluid are added three to four volumes of 95 per cent alcohol and the resultant precipitate is washed with alcohol 95 per cent, absolute alcohol and ether.

Talbot (12) referred to the "brilliant investigations of Oscar M. Schloss." This author applied the skin test according to Schloss.

In 1910 Moss (7) urged the use of a cutaneous test before the reinjection of antitoxic serum and in the test he employed an intradermal injection method injecting 0.01 cc. of undiluted serum. The reactions, which he observed, occurred at considerable intervals after the injections (ten to twenty-four hours) and were, therefore, different from the immediate reactions seen in the natural allergies. On account of the limited scope of this investigation, the technic adopted by Moss did not become generally known nor used.

In 1911, Robert A. Cooke (3, 4), began his independent studies in allergy. From the beginning Cooke's chief purpose was the application of the principle of desensitization in the prevention and treatment of the symptoms of allergy and as this involved the injection of the substance, it was convenient to use the latter in liquid form. It was convenient, also, in using the liquid preparations for the skin test, to apply them by the intradermal or injection method, and this method was adopted by Cooke and employed by him and his assistants and pupils to the present time. This method is the same as that used afterwards by Schick in his well known test of immunity to diphtheria.

Thus there are in use at present two different methods of testing the skin in allergic conditions, the method of Schloss, which we shall refer to as the cutaneous or scratch test and the method of Cooke, which may be designated as the intradermal or injection test.

Regarding the relative efficiency of the two methods Schloss (9) expresses the opinion that the intradermal test is more sensitive than the cutaneous, but thinks the former is "apt to be misleading. Pseudo-reactions occur which are difficult to interpret, ... On two occasions I have seen severe infections due to such tests."

I. C. Walker and J. Adkinson (16) have attempted a comparison of the two methods. From the published report of their experiments it is evident that these authors were seriously handicapped by a lack of acquaintance with the proper method of applying the intradermal test and by inexperience in interpreting the reactions produced by this test. They injected 0.1 cc., which is five to ten times the volume of fluid necessary for this mode of application and they arbitrarily considered that "any appreciable increase over these normal measurements (of the swelling produced mechanically by this large injection) means a positive reaction."

This unfortunate circumstance vitiates all of their comparative experiments and consequently nullifies their conclusions. Incidentally, one of these conclusions is that the intradermal test is "much too sensitive, therefore it is not an index to proper treatment." In this connection it may be observed; first, that there is no need for such an index; secondly, that the intradermal reaction properly applied is distinctly modified by specific treatment in a way that could be used if it were necessary and thirdly, that the advantage claimed for the scratch test by the authors mentioned depends, as we shall show, upon a serious defect in that test—a defect evidenced by the fact that it produces a positive reaction only in the more sensitive individuals. In other words, after treatment has been carried to a certain point the scratch test is no longer able to produce a positive reaction. In the present communication it is intended to report the results of comparative skin tests carried out on the same individuals with the two methods, and as different materials were used, the investigation also compared the dry extracts with the fluid preparations.

TECHNIC

The intradermal or injection test

The fluid preparations used for these tests were obtained from the Department of Applied Immunology in the New York Hospital. The methods of preparing these materials are described elsewhere in this issue (2). The fluid preparations of horse dander contained 0.04 mgm. of protein nitrogen per cubic centimeter; those of the other materials contained 0.5 miligrams of nitrogen per cubic centimeter.

The injection of the fluid was made with the tuberculin syringe, which is graduated in hundredths of a cubic centimeter. The hypodermic needles were of 26 gauge. The syringe and needles were sterilized by boiling.

The injection was made into the skin on the outer aspect of the upper arm. About 0.01 cc. was injected, forming an elevation about 2 to 3 mm. in diameter. Previous to the injection the skin was cleansed with alcohol.

The results of the intradermal tests were noted five to ten minutes after the injection, the height of the reaction being reached within that time.

The cutaneous or scratch test

These tests were carried out with two different kinds of preparations. One was in the form of a powder supplied by a commercial organization and obtained in the open market. The other was the same as those used for the intradermal tests.

The dry preparations were used as follows: An abrasion or scratch $\frac{1}{8}$ inch (3 mm.) long was made with a von Pirquet borer, on the flexor surface of the forearm. The abrasion was not made deep enough to draw blood. A small quantity of the powder was applied to the abrasion, moistened with a drop of decinormal sodium hydrate solution and rubbed into the scratch. For the purpose of control a similar abrasion was made into which only the decinormal alkali was rubbed. After twenty to thirty minutes the test mixture was washed off with 50 per cent alcohol and the result was noted.

When the scratch test was carried out with the fluid preparations, a drop of the latter was rubbed into the abrasion, without addition of sodium hydrate. In these cases the control tests were made with sterile normal salt solution. The results of these tests were noted after five to twenty minutes.

A positive reaction in both tests was determined by the formation of a wheal with an area of erythema surrounding it. To be of diagnostic significance the wheal must be at least 0.5 cm. in diameter. If the wheal was 1–2 cm. in diameter the reaction was given the designation of + +; if the diameter of the wheal exceeded 2 cm. the reaction was designated + + +. In the larger reactions irregularity of outline of the wheal was noted and "pseudopod" formation, which was more common and more marked with the intradermal test.

The comparative tests were carried out with the following preparations:

Animal emanations. Horse, dog, cat, rabbit, goose.

Foods. Egg white, wheat, milk albumin, casein.

The individuals upon whom the comparative tests were performed were all subject to asthma.

The causal relationship of the respective material to the asthmatic symptoms in each case was established on two or more grounds, which are enumerated below and designated in the tables by the corresponding numbers.

1. Symptoms appear after contact with or upon proximity to the original material.
2. Asthmatic symptoms develop after a diagnostic injection.
3. Asthmatic symptoms disappear after a course of therapeutic injections.
4. Symptoms disappear upon avoidance of or removal of the original material.

It will be seen that in every case the intradermal test with the respective protein resulted positively. Objection may be made to our selection of cases on the ground that it was made with reference to the result of the intradermal test. We can only reply to such objections, that with the most conscientious observation of the cases presenting themselves, we have encountered

among those clinically sensitive to the proteins used in this comparative study, none in which the intradermal tests with the respective protein resulted negatively. Our experience with most fruit juices has been different from this. With these preparations, we have observed some negative reactions in individuals that were clearly sensitive to the original material.

The results of the comparative tests are presented in tables 1 to 6.

TABLE 1

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Horse dander	Horse epithelium		
		Scratch test	Intradermal	Scratch	
1	2686 C	+	++	Not done	1, 3 and 4
2	61181c	+	+++	+	1 and 4
3	67090c	++	+++	++	1, 3 and 4
4	67250c	+	+++	++	1 and 4
5	65423c	++	++	++	1, 3 and 4
6	67602c	+	+++	++	1 and 4
7	68146c	++	+++	++	1, 3 and 4
8	66140c	+	+++	++	1, 3 and 4
9	70446c	+	+++	++	1 and 4
10	69670c	+	+++	++	1 and 4
11	2711 C	−	+++	++	1, 3 and 4
12	581 C	−	+++	++	1, 3 and 4
13	67351c	−	+++	++	1, 3 and 4
14	67787c	−	+++	++	1, 3 and 4
15	66767c	−	+++	++	1 and 4
Summary	15	Positive 10 Negative 5	Positive 15	Positive 14	

The individual tables need no explanatory comment. The results in each series were concordant in the sense that:

1. The intradermal test is superior to the cutaneous or scratch test.

2. The fluid preparations made according to the methods of Coca are superior to the corresponding dry commercial preparations.

TABLE 2

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial powder	Fluid preparations (Coca)		
		Dog hair	Dog epithelium		
		Scratch test	Intradermal	Scratch	
1	2686 C	+	+++	+++	1 and 4
2	61181c	+	+++	++	1 and 4
3	67090c	++	+++	++	1 and 4
4	65423c	+	+++	++	1 and 4
5	66804c	+	++	++	1 and 4
6	69670c	+	+++	++	1 and 4
7	2691 C	–	+++	Not done	1, 3 and 4
8	67351c	–	+++	++	1 and 4
9	2782 C	–	+	+	1 and 4
10	18B	–	+++	–	1 and 4
11	67096c	–	+++	–	1 and 4
12	61B	–	+++	++	1, 3 and 4
Summary	12	Positive 6 Negative 6	Positive 12 Negative 0	Positive 9 Negative 2	

TABLE 3

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Rabbit hair powder	Rabbit epithelium		
		Scratch test	Intradermal	Scratch	
1	67250c	+++	+++	++	1 and 4
2	66761c	+	++	++	1 and 4
3	66769c	+	++	+	1 and 4
4	68146c	++	+++	++	1 and 4
5	66804c	++	+++	++	1 and 4
6	63B	+	+++	+	1 and 4
7	2713C	–	+++	+	1 and 4
8	69670c	–	+++	–	1 and 4
9	48B	–	+++	–	1, 3 and 4
Summary	0	Positive 6 Negative 3	Positive 9 Negative 0	Positive 7 Negative 2	

TABLE 4

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Goose feathers powder	Goose epithelium		
		Scratch test	Intradermal	Scratch	
1	2713 C	++	+++	Not done	1 and 4
2	61181c	++	+++	++	1, 3 and 4
3	67090c	++	+++	++	1 and 4
4	67250c	++	+++	++	1 and 4
5	66804c	+	++	++	1 and 4
6	2648 C	−	+++	Not done	1 and 4
7	1949 C	−	+++	Not done	1 and 4
8	67351c	−	+++	++	1 and 4
9	66769c	−	+++	++	1 and 4
10	67602c	−	+++	++	1 and 4
11	67787c	−	+++	+	1 and 4
12	2782 C	−	+	++	1 and 4
13	68146c	−	+++	+	1 and 4
14	66146c	−	+++	+	1 and 4
Summary	14	Positive 5 Negative 9	Positive 14 Negative 0	Positive 11 Negative 0	

TABLE 5

NUMBER	CASE	ANIMAL EMANATIONS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Cat hair powder	Cat epithelium		
		Scratch test	Intradermal	Scratch	
1	2739 C	+	+++	+	1, 3 and 4
		−	+++	+	1, 3 and 4*
2	61181c	++	+++	+	1 and 4
3	67090c	+	+++	++	1 and 4
4	66761c	+	+++	++	1 and 4
5	67351c	+	+++	++	1 and 4
6	66995c	+	+++	++	1 and 4
7	67787c	+	+	+	1 and 4
8	2782 C	+	+++	++	1 and 4
9	70446c	+	+++	++	1 and 4
10	2601 C	−	+++	Not done	1 and 4
11	2711 C	−	+++	−	1, 3 and 4
		−	+++	−	1, 3 and 4*
12	2713 C	−	+++	−	1, 3 and 4
13	60670c	−	+++	++	1 and 4
14	15B	−	+++	++	1, 2, 3, and 4
15	67096c	−	+++	−	1 and 4
Summary	17	Positive 9 Negative 8	Positive 17 Negative 0	Positive 12 Negative 4	

* Test 2 weeks later.

TABLE 6

NUMBER	CASE	FOOD PROTEINS			RELATION OF ORIGINAL MATERIAL TO SYMPTOMS ESTABLISHED BY
		Commercial	Fluid preparations (Coca)		
		Egg white powder	Egg white		
		Scratch test	Intradermal	Scratch	
1	1949C	++	+++	Not done	1 and 4
2	66360c	+++	+++	++	1 and 4
3	2671C	+	+++	Not done	1 and 4
4	2699c	–	+++	Not done	1 and 4
			Wheat		
5	70248c	–	+	+	1, 3 and 4
		–	+	–	1, 3 and 4*
6	69839c	–	+++	++	1, 3 and 4
7	70831c	–	+++	–	1, 3 and 4
8	61B	–	+++	–	1 and 4
			Milk albumin		
9	1949c	–	+++	Not done	1 and 4
			Casein		
10	1949c	–	+++	Not done	1 and 4
Summary	11	Positive 3 Negative 8	Positive 11 Negative 0	Positive 3 Negative 4	

* Test 2 weeks later.

SUMMARY TABLES 1–6

	COMMERCIAL POWDERS SCRATCH METHOD				FLUID PREPARATIONS INTRADERMAL METHOD				FLUID PREPARATIONS SCRATCH METHOD			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
1	5	7	3	0	0	0	2	13	0	1	13	0
2	6	5	1	0	0	1	1	10	2	1	7	1
3	3	3	2	1	0	0	2	7	2	3	4	0
4	9	1	4	0	0	1	1	12	0	3	8	0
5	8	8	1	0	0	1	0	16	4	4	8	0
6	8	1	1	1	0	2	0	9	4	1	2	0
Summary	39	25	12	2		5	6	67	12	13	42	1

The first conclusion is drawn from a comparison of the middle and right hand columns of the summary of the tables. It is seen that when fluid preparations were used, the reaction was positive in all of the 78 cases with the intradermal method, but negative in 12 of the cases with the scratch method.

The second conclusion is drawn from a comparison of the left hand and right hand columns of that summary. When the scratch method was used with the fluid preparations the reaction was negative in 12; that is, about 18 per cent of the cases tested. With the commercial powders the scratch method resulted negatively in 39 cases or 50 per cent of those tested.

The two methods of applying the skin tests must be compared in other respects besides these that we have already discussed.

1. Relative ease of performing the tests

The scratch method has been preferred by some because it was thought to be more easily applied than the intradermal method. Our own experience with the two procedures has satisfied us that the reverse is true.

As it is often necessary to carry out the skin tests with a number of different substances as well as with different dilutions of the same substance, the time required for the test is a practically important consideration, and moreover the time factor may be taken as a fair index of the relative ease with which the two methods of testing can be applied.

We have carried out a series of 52 parallel tests in the same individuals, with the purpose of determining the relative time required for the application of the two methods. The fluid preparations of Coca were employed for both methods. This was done for the following reasons.

The fluid preparations can be applied more quickly with the scratch technic, than the dry products and the reaction with the fluid preparations reaches its maximum sooner than that with the dry products. Moreover, as we have shown above, the cutaneous reaction is generally more marked with the fluid preparations, than with the dry ones. The data obtained from these parallel tests are presented in table 7.

The results of these comparative tests demonstrate that even with the more convenient and more active fluid preparations over 50 per cent more time is required for the development of a positive reaction with the scratch technic than is needed with the injection method.

TABLE 7

REACTION TIME WITH THE INTRADERMAL METHOD		REACTION TIME WITH THE SCRATCH METHOD	
10 minutes	7 minutes	10 minutes	14 minutes
8 minutes	10 minutes	11 minutes	9 minutes
10 minutes	6 minutes	10 minutes	13 minutes
10 minutes	10 minutes	10 minutes	10 minutes
7 minutes	5 minutes	10 minutes	9 minutes
10 minutes	5 minutes	7 minutes	17 minutes
7 minutes	10 minutes	15 minutes	14 minutes
11 minutes	7 minutes	8 minutes	7 minutes
5 minutes	6 minutes	13 minutes	9 minutes
10 minutes	4 minutes	7 minutes	7 minutes
5 minutes	9 minutes	14 minutes	18 minutes
7 minutes	10 minutes	7 minutes	10 minutes
7 minutes	5 minutes	14 minutes	8 minutes
5 minutes	8 minutes	15 minutes	17 minutes
4 minutes	4 minutes	22 minutes	10 minutes
11 minutes	9 minutes	10 minutes	13 minutes
10 minutes	6 minutes	10 minutes	11 minutes
6 minutes	5 minutes	7 minutes	8 minutes
5 minutes	8 minutes	12 minutes	11 minutes
10 minutes	4 minutes	14 minutes	10 minutes
10 minutes	5 minutes	5 minutes	12 minutes
5 minutes	5 minutes	12 minutes	14 minutes
10 minutes	5 minutes	10 minutes	11 minutes
6 minutes	6 minutes	7 minutes	8 minutes
5 minutes	7 minutes	17 minutes	13 minutes
9 minutes	8 minutes	12 minutes	9 minutes
Average reaction in $7\frac{1}{4}$ minutes Earliest reaction in 4 minutes Latest reaction in 11 minutes		Average reaction in $11\frac{2}{5}$ minutes Earliest reaction in 5 minutes Latest reaction in 22 minutes	

Less time is needed also in applying the intradermal test, than is required for the scratch test. With an adequate supply of sterile syringes 20 intradermal tests can be made in five minutes. With the dry commercial preparations the 20 tests can hardly be made in less than thirty minutes.

It is advised by the manufacturers of the dry preparations to make the "readings" after thirty minutes. In a large clinic or in an active private office, the shorter time needed for applying and reading the intradermal tests, weighs heavily in favor of this method.

2. Relative convenience to the patient

With the intradermal method a minimal amount of damage of damage is inflicted on the skin—merely the prick of a sharp, fine hypodermic needle. With the scratch method an incision $\frac{1}{8}$ inch long is made or an area of equal diameter is abraded by scraping. The rubbing of the test mixture into the traumatized skin does further damage. We believe that the intradermal method is the less painful of the two. The marks of the injection method usually disappear within a week; those resulting from the scratch method often persist for weeks or even for months.

3. Greater convenience of the fluid preparations

Considerable convenience is afforded in the use of the fluid extracts by the fact that the same preparation is used for diagnosis and for treatment. This advantage of the fluid extracts over the dry preparations becomes more important as the number of preparations used increases.

4. Constitutional reactions

All other factors being equal, the quantity of material absorbed after its injection should be greater than after its application to an abraded surface. Hence it should be expected that constitutional reactions would be more frequent with the intradermal method than with the scratch method. The writer is unable to say whether this is actually true. The experiences of Rufus Cole cited above and of Cooke (5) demonstrate that constitutional reactions may result from either the scratch method or the intradermal method.

With regard to the possibility of infection resulting from the intradermal injection, which was mentioned by Schloss, we can only say that with ordinary aseptic precautions in the preparation of the extracts and in their use, there is no danger of infection. In the writer's experience, which is in agreement with that of Cooke and that of Vander Veer, infection would occur only as a result of gross disregard of the simple principles of ordinary asepsis.

SUMMARY AND CONCLUSIONS

Two methods of applying the skin test in allergic conditions are in use:

1. The cutaneous or scratch method of Schloss.
2. The intradermal or injection method of Cooke.

Two forms of test proteins are in use:

1. The dry powdered preparations made according to the methods described by Wodehouse.
2. The fluid preparations originally used by Cooke and made now according to the methods described by Coca.

In a series of 78 comparative tests the superiority of the intradermal method over the scratch method has been shown upon the following grounds:

1. In every case known to be clinically sensitive to a protein, the intradermal test with that protein resulted positively. The scratch test with the corresponding dry preparations resulted positively in only half the cases tested. The scratch test with the fluid preparations resulted negatively in 18 per cent of the cases tested.
2. The intradermal method properly applied is not so painful as the scratch method and the resulting markings of the skin do not persist so long after the former method.
3. Less time is required for applying the intradermal method and for obtaining the results than is needed for the scratch method.
4. The same preparation can be used for testing and for treatment when the fluid preparations are employed.

REFERENCES

(1) Blackley, C. H.: Exper. Researches on Causes and Nature of Hay Fever, 1873.
(2) Coca, A. F.: Jour. of Immunol., 1922, **7**, 163.
(3) Cooke, R. A.: Laryngoscope, 1915, **25**, 108.
(4) Cooke, R. A., and Vander Veer, A., Jr.: Jour. of Immunol., 1916, **1**, 201.
(5) Cooke, R. A.: Jour. of Immunol., 1922, **7**, 119.
(6) Goodale, J. L.: Boston Med. and Surg. Jour., 1916, **175**, 181.
(7) Moss, W. L.: Journ. Amer. Med. Assoc., 1910, **55**, 776.
(8) Schloss, O. M.: Amer. Jour. Dis. of Child., 1912, **3**, 341.
(9) Schloss, O. M.: Amer. Jour. Dis. of Child., 1920, **19**, 433.
(10) Schmidt, H.: Arch. f. Kinderhlk., 1910, **53**, 349.
(11) Smith, H. L.: Arch. Int. Med., 1909, **3**, 350.
(12) Talbot, F. B.: Boston Med. and Surg. Jour. 1914, **171**, 708.
(13) Walker, I. C.: Jour. Med. Research. 1917, **35**, 487.
(14) Walker, I. C.: Boston Med. and Surg. Jour., 1918, **179**, 288.
(15) Walker, I. C.: Asthma. Oxford University Press, 1920.
(16) Walker, I. C., and Adkinson, J.: Journ. Med. Res., 1917, **37**, 287.
(17) Wodehouse, R. P.: Boston Med. and Surg. Journ., 1916, **175**, 195.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

II. A COMPARISON OF VARIOUS POLLEN EXTRACTS WITH REFERENCE TO THE QUESTION OF THEIR THERAPEUTIC VALUE IN HAY FEVER

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The following study of the relative strengths of various commercial extracts used in the treatment of hay fever was made because there is no recognized standard method of preparation of such extracts and it was felt that it might be of interest to see what therapeutic results might reasonably be expected from their use. Preparations of four of the best known commercial products were purchased from a pharmacy and compared with corresponding extracts obtained from the Department of Applied Immunology in the New York Hospital. The method of preparation of these latter extracts is published by Dr. A. F. Coca in this issue of the Journal of Immunology (1). The commercial products have been designated A, B, C, and D, while those made in the New York Hospital are labelled E. For convenience the last named extracts will be referred to as the "Cornell preparations." All of the five preparations were in fluid form. Three strengths (designated 1, 2 and 3, in order of strength) were tested of preparation A, three of B, two of C, two of D and three of E. Preliminary tests showed that only the very sensitive cases gave any ophthalmic reactions to the preparations A, B, C and D, hence our study was necessarily limited to a relatively small group. Eighteen cases in all were used for this purpose but they were not all tested for each preparation and each strength, since it is not practicable to test with more than three or four extracts at

TABLE 1

HISTORY NUMBER	A						B						C				D				E					
	1		2		3		1		2		3		1		2		1		2		1		2		3	
	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye	skin	eye
2581							0	0	+	0	+	0									+++	0	+++	+		
3193	+++	++					0	0	+	0	+++	++	+	0	+++	++	++	++			+++	+	+++	+	+++	++
3166	+++	+					0	0	+++	0	+++	0					+++	++			+++	0	+++	+	+++	++
3144	+++	0					0	0	+++	0	+++	0	+++	0	+++	+	+++	0	+++	0	+++	0	+++	+	+++	++
3207																	+++	0	+++	+			+++	0	+++	+
2001																	+++	0	+++	0			++	0	+++	+
3208											++	0					++	0	+++	0					+++	+
3121	+++	0				0							0	0	++	0							+++	0		
3110													0	0	+++	0	+++	0	+++	0			+++	0	+++	+
2534	+++	0			+++	+	0	0	+++	0	+++	+	+++	0	+++	+							++	+	+++	++
1156									+	0	+	0							++	+			+++	+	+++	+
3245					+++	+	0	0	+++	+			+++	+							+++	0	+++	+	+++	++
2865	+++	0	+++	0	+++	+			++	0	+++	0											+++	+		
2273	+++	0					+	0	+	0	+++	0									+	0	+++	+++		
1550			++	0	+++	0	0		0	0	+++	0			++	0	+	0	0	0	+++	+	+++	+		
2990												+				0					+	0	+++	+++		
1409			+++	0	+++	+															++	0	++	+		
183																	+++	+	+++	+	+++	0	+++	+	+++	+

one sitting. This is particularly true if a positive ophthalmic reaction is obtained with one of the first extracts. In this event, no more tests can be made in this eye at that time. As the ophthalmic reaction often becomes less pronounced after a few therapeutic injections the same patient cannot be tested with other extracts at a later date for comparison, unless it is determined that such a diminution in sensitiveness has not taken place. This could often be determined by testing one eye with the same strength of the Cornell preparation which had given a reaction on a previous testing. If the reaction was the same it was fair

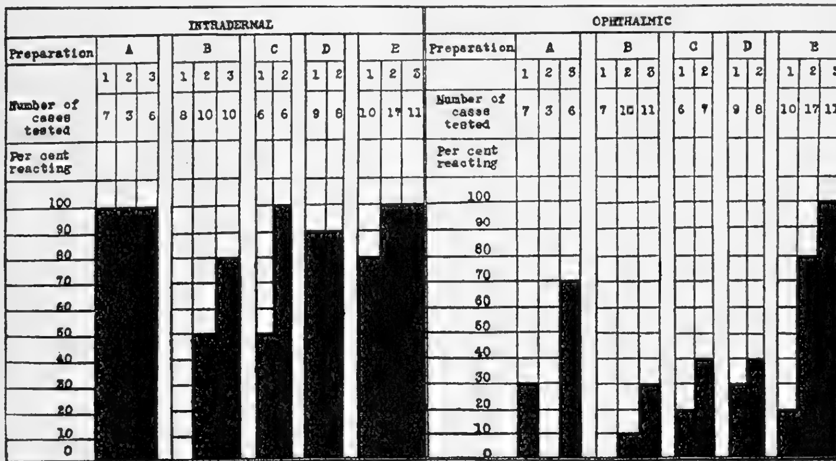

CHART 1

to assume that no diminution in sensitiveness had taken place and the other eye was then used for further testing. All of the eighteen cases were tested with one or more of the Cornell preparations, while from six to ten cases were tested with each of the other extracts (with the exception of preparation A2 to be mentioned below). The results are tabulated in table 1 and a graphic representation of these results is given in chart 1. The following symbols are used to designate reactions; 0 = negative; + = slight; ++ = moderate; +++ = marked. In the graphic chart 0 and + are considered negative reactions, while ++ and +++ are positive reactions for the intradermal tests. For the

ophthalmic tests, 0 indicates a negative and +, ++, and +++ indicate positive reactions.

The strength of the various preparations is said by the producers to be as follows:

A1 = 1000 pollen units; A2 = 1250 pollen units; A3 = 1500 pollen units.

B1 = 10 pollen units; B2 = 100 pollen units; B3 = 1000 pollen units.

C1 = 0.02 mgm. of nitrogen to 1 cc.; C2 = 0.08 mgm. of nitrogen to 1 cc.

D1 = 1:1000 dilution; D2 = 1:100 dilution.

E1 = 0.001 mgm. of nitrogen to 1 cc., E2 = 0.005 mgm., E3 = 0.01 mgm. of nitrogen to 1 cc.

The methods of determining the value of the pollen units in preparations A and B; of determining the nitrogen in preparation C and of making the dilution in preparation D are not known to the author. The nitrogen in preparation E was determined by the Kjeldahl method.

In comparing the results of the tests both the intradermal and the ophthalmic reactions were considered but as the eye is less sensitive than the skin (by the intradermal test) and therefore requires a stronger solution to give a positive reaction the difference in the strengths of the solutions is more clearly demonstrated in the chart of the ophthalmic reactions. A résumé of these results shows that (with one exception to be noted later) rough comparisons may be drawn between A, B, C, and D, with E as follows: The activity of preparation A1 is equal to that of preparation E1; the result with preparation A2 must be disregarded because only three cases were tested with this extract and, while these all gave positive intradermal reactions, they all gave negative ophthalmic reactions; (it happened that among the group of eighteen tested these three individuals were comparatively insensitive and it is probable that if five or six more cases had been tested enough of them would have reacted with A2 to give a higher percentage of ophthalmic reactions); the activity of preparation A3 is equal to that of preparation E2; preparation B1 was apparently inert as it produced no intrader-

mal or ophthalmic reaction in the eight cases tested; preparation B2 was less active than preparation E1; the activity of preparation B3 is equal to that of preparation E1; the activity of preparation C1 is equal to that of preparation E1; preparation C2 was little stronger than preparation E1; preparations D1 and D2 caused nearly equal reactions; both were a little stronger than preparation E1.

No commercial preparation even approximated the strength of preparation E3 (0.01 mgm. of nitrogen to 1 cc.) and the activity of preparation A3 (the strongest of A, B, C, or D) was about equal to that of preparation E2 (0.005 mgm. of nitrogen to 1 cc.). As it has been our experience that even our most sensitive cases (with very few exceptions) require for therapeutic effect a maximum dose of pollen extract containing at least 0.025 to 0.05 mgm. of nitrogen while the less sensitive may need as high as 0.1 or 0.2 mgm. of active pollen nitrogen, it is difficult to see how a good result can be expected with the use of these comparatively weak commercial preparations except in the very sensitive cases, which constitute a relatively small percentage of the total. To obtain a full measure of relief stronger extracts should be used; however, if this is done, more caution must be exercised in their use as the more concentrated extracts are more apt to cause constitutional reactions (2).

The necessity for adequate dosage is well illustrated by the history of case 2273. This patient came to us in 1920 saying that he had been treated during 1919 with commercial preparation A for both early and late hay fever. The early hay fever was almost entirely relieved but the late hay fever was unaffected by the treatment. Unfortunately the first part of his history containing the record of his tests in 1920 has been lost. However, in 1921, he gave a positive eye reaction to an extract of timothy pollen (Cornell) containing 0.005 mgm. of nitrogen to 1 cc. while with ragweed pollen extract (Cornell) no eye reaction was produced by concentrations less then 0.1 mgm. of nitrogen in 1 cc. The ratio of his sensitiveness to the pollens, according to these tests was, thus, 20 to 1. In 1920 he received injections of commercial preparation A for his early hay fever and a Cornell extract of ragweed for his late hay fever.

The largest dose (in terms of nitrogen) of the latter preparation that he received was 0.16 mgm. Since our comparative tests indicate that the strongest solution of preparation A contained only about 0.005 mgm. of active nitrogen per cubic centimeter, it can be estimated that the patient received 30 times as much active ragweed pollen protein in 1920 as he had received in 1919. The results of the treatment were correspondingly different, for the injections of the Cornell extract of ragweed pollen produced the same degree of relief from the late hay fever as had been attained for the early hay fever with preparation A. During 1921 he received the Cornell extracts for both early and late hay fever, doing equally well with both; his maximum dose (in nitrogen) for the season was 0.04 mgm. of timothy pollen while his maximum dose of ragweed pollen was 0.16 mgm.

This case plainly illustrates that patients vary markedly in their degree of hypersensitiveness and also in the size of the dose necessary to relieve their symptoms.

Since this paper makes a plea for the use of larger therapeutic doses and more potent agents, it does not seem amiss again to caution the reader that this is attended with greater risk of producing constitutional reactions which in inexperienced hands can be dangerous (2).

REFERENCE

(1) Coca, A. F.: Jour. Immunol., 1922, 7, 163.
(2) Cooke, R. A., Journ. of Immunol., 1922, 7, 119.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

III. ON CONSTITUTIONAL REACTIONS: THE DANGERS OF THE DIAGNOSTIC CUTANEOUS TEST AND THERAPEUTIC INJECTION OF ALLERGENS

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DEFINITION OF THE TERM

The term constitutional or general reaction is used to designate the group of symptoms occurring in allergic individuals after the absorption of an allergen and its transportation by the blood and lymph into the systemic circulation. Symptoms therefore occur in various organs and tissues affected by the allergen and may be protean in nature, since they depend upon the structures involved, which may differ with the individual and the allergen concerned. Such reactions may take place when the allergen is introduced through unnatural channels, as in the diagnostic skin test, subcutaneous or intravenous injection, or through natural channels, as after ingestion.

DATA USED

The data for the present paper are obtained more especially from a statistical study of 578 consecutive cases observed in 1920 and somewhat more generally from a personal experience over a period of ten years in the diagnosis and treatment of some four thousand allergic cases. The appended protocol gives the synopsis of the 61 cases with constitutional reactions occurring in 1920. Table 1 includes the facts relating to the 578 cases of that year.

TABLE 1

Summary of allergic cases studied in 1920

Number of cases	578
Undiagnosed	105
Allergic cases	473
Number of tests on allergic cases	13,576
Allergic cases giving constitutional reaction on test or injection (see protocol)	61
Allergic cases giving constitutional reaction (all immediate) on test	10
Number of constitutional reactions on test	11
Allergic cases therapeutically injected	414
Number of injections	5,416
Cases with immediate constitutional reactions on injection	31
Number of immediate constitutional reactions on injection	42
Cases with delayed constitutional reaction on injection	20
Number of delayed constitutional reactions on injection	44
Cases with constitutional reaction on injection, onset time unknown	6
Number of constitutional reactions on injection, onset time unknown	8
Summary of pollen allergies	
Cases positive to pollen extract on test	393
Cases with constitutional reaction to pollen extract on test	3
Cases of pollen allergy therapeutically injected	310
Number of pollen extract injections given	4,215
Cases of pollen allergy with immediate constitutional reaction on injection	28
Number of immediate constitutional reactions to pollen extract on injection	38
Cases of pollen allergy with delayed constitutional reaction on injection	15
Number of delayed constitutional reactions to pollen extract on injection	38
Cases of pollen allergy with constitutional reaction on injection, onset time unknown	5
Number of constitutional reactions on injection with pollen extract, onset time unknown	6

No previous attempt has been made seriously to consider this subject in its important relation to, and as a consequence of, the diagnostic study and the treatment of hypersensitiveness in

the human subject. It is the object of this paper to present and to correlate all the facts relating to such general reactions, as this type of work is being, and will continue to be, much more generally used and especially because of the dangerous and sometimes fatal results which may occur.

In all of these cases the diagnostic tests were made by the intradermal method and a definite diagnosis of allergy was based upon a marked cutaneous reaction: that is, a wheal with obvious pseudopod formation surrounded by a definite zone of hyperemia. It was further required that this marked positive reaction be repeatedly confirmed. The preparations used were those prepared by the writer standardized according to the nitrogen content. There are no data with which to compare the relative frequency of general reactions occurring with the hypodermic as opposed to the scratch method of cutaneous diagnostic test. It can only be pointed out that reactions do occur with the latter method, as, for example, the reaction produced by Rufus Cole with buckwheat, reported by Smith (1), using the scratch test. Some years ago the writer witnessed a constitutional reaction to timothy pollen where the pollen was applied to an abrasion on the forearm and dissolved in 0.8 per cent salt solution. Within five minutes there was a local reaction with a very difinite lymphangitis extending to the axilla, with intense itching in the cubital fossa and axilla. This was almost immediately followed by the general symptoms of erythema, urticaria, general pruritus, asthma and coryza, characteristic of the general allergic reaction. This lymphangitis is very characteristic of all marked positive reactions obtained on the anterior aspect of the forearm, even in the absence of general symptoms, and gives a clue as to the part the lymphatics generally must take in all local or general reactions.

It may be that constitutional reactions occur more frequently after the intradermal than after the scratch test. Since the general reaction depends solely upon the amount of the active allergen absorbed, it is possible that if such a difference does exist it will be found to be due to the use, in the intradermal test, of a more potent extract than is generally used in the scratch

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions fr m	Onset of symptom	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2166			Orris	Orris			
2176		Timothy					
2177			Dust, cat				
2180			Horse	Horse			
2185		Ragweed	LePage	LePage	LePage	Immediate	Asthma, coryza, caria
2193		Timothy					
902		Ragweed		Orris, horse and dog epi thelium			
2197		Timothy					
2199		Timothy					
2213		Timothy					
2388		Timothy					
2393	Ragweed	Timothy Ragweed		Orris			
2415	Ragweed	Timothy Ragweed					
2416		Ragweed					
2428	Ragweed	Ragweed					
2440		Ragweed					
2441		Ragweed					
2447			Horse				
2473	Ragweed	Ragweed		Orris, horse			
2479	Ragweed	Ragweed Timothy	Orris		LePage	Immediate	Urticaria
2492	Ragweed	Ragweed					
2520		Ragweed					
2526			Ipecac Linseed Buckwheat Feathers	Ipecac Linseed Buckwheat Feathers	Linseed	Immediate	Asthma, coryza, caria. Angio-n tic edema of 1 lasting 2 days

PROTOCOL

ON SUBCUTANEOUS INJECTION					PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
stitutional reactions from	Injection reacting	Onset of symptons	Duration of symptoms	Symptoms		
ris	4th	8 hours	2-3 days	Asthma	?	Marked
	5th	8 hours	10 hours	Asthma	?	Marked
nothy	10th	1 hour	3 hours	Cough and urticaria	?	None
t epithelium	5th	8 hours	?	Asthma	?	?
rse epithelium	1st	Immediate	6 hours	Asthma, coryza, urticaria, also nausea and headache lasted one week	Patient very sensitive	Marked
Page	1st	Immediate	24 hours	Asthma, coryza, urticaria	Patient very sensitive	Marked
nothy	8th	8 hours	12 hours	Pruritus of arms and neck	Change of extract	Marked
gweed	8th	12 hours	8 hours	Coryza, cervical glands	?	Slight
	9th	12 hours	8 hours	Coryza, cervical glands	?	Slight
nothy	10th	Immediate	10 days	Asthma, coryza, cervical glands	Concentrated extract	?
nothy	8th	Immediate	?	Urticaria	Concentrated extract	?
mothy	5th	Immediate	4 hours	Coryza, erythema, headache and pruritus	Dose increased too rapidly	Moderate
mothy	7th	1 hour	1 day	Asthma, coryza	?	Moderate
	10th	Immediate	1 hour	Asthma, coryza	?	?
gweed	17th	6 hours	2 days	?	Overdose	?
gweed	16th	?	1 day	Coryza	?	Marked
gweed	12th	24 hours	1 day	Coryza and edema of lip	Change of extract	None
gweed	14th	1 hour	?	Asthma, coryza, urticaria	Change of extract	?
gweed	16th	24 hours	6 days	Coryza, headache	?	?
gweed	18th	?	?	Coryza, edema of eyes	?	?
rse epithelium	9th and 10th	?	?	Asthma	?	?
rse epithelium and	10th	4 hours	2 days	Coryza	?	?
ragweed	11th	4 hours	2 days	Coryza	?	?
gweed					Patient very sensitive	Marked
gweed	10th	1 hour	3 hours	Asthma	Change of extract	Slight
	17th	½ hour	?	Asthma	Change of extract	?
gweed	7th	Immediate	?	Asthma	?	Moderate
	10th	Immediate	?	Asthma	?	?
					Patient very sensitive	Marked

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions from	Onset of symptoms	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2274	Timothy	Timothy					
2276		Ragweed					
2283		Ragweed					
2284	Timothy	Timothy					
2285		Timothy					
		Ragweed					
2286	Ragweed	Ragweed	Peach	Peach			
	Daisy	Daisy	Celery	Celery			
	Dandelion	Dandelion	Raspberry	Raspberry			
2290	Ragweed	Ragweed	Horse, cat	Horse, cat			
			Feathers, dust	Feathers			
2292		Ragweed		Orris			
2327		Ragweed					
2350	Ragweed	Ragweed					
2351		Ragweed					
2364		Ragweed	Horse				
2381	Ragweed	Ragweed	Horse	Horse			
2365	Timothy	Timothy					
2386		Timothy					
2367		Timothy					
2370		Ragweed					
2214	Ragweed	Ragweed	Feathers				
2216		Timothy					
2227		Timothy					
		Ragweed					
2233		Timothy					
2643		Timothy		Orris; dust			

PROTOCOL

ON SUBCUTANEOUS INJECTION					PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
nstitutional reactions from	Injection reacting	Onset of symptoms	Duration of symptoms	Symptoms		
mothy	5th	Immediate	3 hours	Coryza	Change of extract	?
agweed	21st	?	?	Asthma, coryza, urticaria	Change of extract	?
agweed	7th	Immediate	?	Coryza	Directions not followed. Intervening doses omitted	?
mothy	14th	Immediate	?	Asthma	Concentrated extract	?
agweed	18th	Immediate	?	Asthma, coryza, urticaria	Change of extract	?
aisy, dandelion	2nd	Immediate	1 hour	Asthma, coryza	Patient very sensitive	Marked
agweed	8th	Immediate	2 hours	Urticaria in all	Patient very sensitive	?
	9th	Immediate	4 hours		Patient very sensitive	?
agweed	9th	Immediate	2-3 hours	Coryza	?	?
	13th	Immediate	2 days	Asthma	?	?
	15th	Immediate	?	Coryza, asthma	?	?
agweed	10th to	6 hours	36 hours	Urticaria	?	?
	20th	Immediate	?	Coryza	Change of extract	?
agweed	15th	Immediate	3 hours	Coryza, asthma, urticaria	Change of extract	?
agweed	19th	?	?	Asthma	?	None
agweed	16th	4 days	4 days	Urticaria	?	Moderate
agweed	18th	2 hours	?	Urticaria	?	Marked
agweed	12th	4 hours	8 hours	Asthma, coryza	Change of extract	Marked
	17th	3 hours	3 days	Urticaria, edema of eyes, coryza	Dose increased too rapidly	?
mothy	11th	Immediate	12 hours	Asthma, coryza, urticaria	Dose increased too rapidly	?
mothy	14th	8 hours	4 hours	Urticaria	?	?
	15th	8 hours	4 hours	Urticaria	?	?
mothy	1st	Immediate	?	Coryza, erythema	Patient very sensitive	Marked
agweed	17th	6 hours	12 hours	Coryza, edema of eyes	Change of extract	Marked
gweed	6th	Immediate	4 hours	Asthma, coryza, urticaria	Concentrated extract	?
	20th	3 days	Several	Urticaria	Change of extract	?
mothy	15th	Immediate	2 hours	Asthma, coryza, urticaria, edema of lips	?	?
	21st	1 hour	2 hours	Urticaria	Change of extract	?
mothy	8th	Immediate	?	Asthma, coryza, urticaria	Concentrated extract	?
gweed	1st	Immediate	?	Severe abdominal pains	Patient very sensitive	Marked
mothy	8th	½ hour	2 days	Asthma, coryza, urticaria, edema face	?	None
	13th	1 hour	8 hours	Asthma, coryza, urticaria, edema face	Change of extract	?
ris	15th	2¼ hours	3 days	Asthma	?	Slight

PROTOCOL

CASE NUMBER	CLINICAL SYMPTOMS				INTRADERMAL TEST		
	Pollens giving		Other substances giving		Constitutional reactions from	Onset of symptons	Symptoms
	Asthma	Coryza	Asthma	Coryza			
2651		Timothy Ragweed	Horse, dog Buckwheat		Ragweed	Immediate	Coryza, asthma Headache, erythema
1058		Timothy			Timothy	Immediate	Coryza
2673			Egg, milk LePage, horse Rabbit, feathers		LePage	Immediate	General edema. Asthma, coryza, death from asphyxia
2681			Orris, wheat Dust		Orris	Immediate	Asthma, urticaria
2684			Dust, feathers				
2258	Ragweed	Ragweed					
2260		Timothy Ragweed					
2273	Timothy Ragweed	Timothy Ragweed					
2547	Ragweed	Ragweed					
2581		Timothy Ragweed					
2627		Ragweed	Orris		Ragweed	Immediate	Coryza, asthma, urticaria
2707			Feathers, dog, cat		Dog saliva	Immediate	Coryza, asthma, urticaria
2708		Timothy Ragweed	Chicken, rabbit Flaxseed, mustard		Flaxseed Mustard	Immediate Immediate	Asthma Asthma
896		Timothy		Cat			
2541	Ragweed	Ragweed					
2532	Ragweed	Ragweed					

test. This surmise is supported by the experiences of Brown (2) and of Larsen, Paddock, and Alexander (3), who found only 50 per cent of diagnostic efficiency using the dry preparation generally employed for the scratch test as compared with the fluid preparations used in the intradermal test.

HISTORICAL

A survey of medical literature discloses the fact that the year 1894, when diphtheria antitoxin was first introduced for general

PROTOCOL

ON SUBCUTANEOUS INJECTION					PROBABLE CAUSE	ASSOCIATED REACTION AT SITE OF INJECTION OR TEST
nstitutional reactions from	Injection reacting	Onset of symptoms	Duration of symptoms	Symptoms		
					Concentrated extract	Marked
orse epithelium	4th	12 hours	2 days	Nausea	?	Marked
					Concentrated extract	Marked
					Patient very sensitive	Marked
					Concentrated extract	Marked
ust extract	5th	1 day	5 days	Urticaria	?	?
agweed	7th	Immediate	?	Asthma	?	None
	10th	Immediate	?	Asthma	?	Marked
agweed	10th	5 days	1 week	Urticaria	Dose increased too rapidly	?
agweed	16th	Immediate	?	Asthma, urticaria	?	?
agweed	2nd	Immediate	?	Coryza, asthma	?	Moderate
agweed	1st	1 hour	2 hours	Urticaria, edema of uvula	Patient very sensitive	?
					Concentrated extract	Marked
					?	?
					Concentrated extract	Marked
					Concentrated extract	Marked
	5th	Immediate	3 hours	Urticaria	?	?
imothy	7th	Immediate	2 hours	Coryza, edema of eyes	Change of extract	?
	12th	Immediate	3 days	Coryza, edema of eyes, headache, urticaria	?	?
agweed	1st	Immediate	?	Coryza, erythema	Patient very sensitive	Marked
agweed	10th	24 hours	1 day	Erythema	?	?

use, marks the beginning of a period in which there are rather frequent reports of the violent reactions and sudden death following the injection of the antitoxic horse serum. The nature of the reaction was not understood. Gottstein (4) reported several such cases in 1896. In 1906, Rosenau and Anderson (5) state, "We have collected from the literature 19 cases of such unfortunate results and know personally of several more which have not been reported." Gillette (6) collected 30 cases Park (7), speaking of the frequency of such general reactions with diph-

theria antitoxin, says that since January 1, 1895, using either whole serum or the globulin fraction, thirty thousand cases had been injected, with collapse in two cases; that over a period of five years, when an immunizing dose was used in all scarlet fever cases, sixteen thousand cases were injected, with no collapse; and that in New York City, inspectors have given injections to one hundred and five thousand cases, with two deaths, but no record was kept of cases with reactions which were not fatal. Every death which did occur followed a primary injection.

The literature since 1913 has not been searched for the antitoxin reactions because the further collection of cases does not add materially to our knowledge of the reaction and because comparatively few of the reactions that have occurred have been reported. We are not interested here in the relative frequency of the allergic reaction to diphtheria antitoxin. Gillette (6) appears to have been the first to remark the similarity of the clinical reaction to the experimental anaphylactic reaction in the guinea-pig and he further noted the important fact that in man most of the severe reactions with dyspnea, edema, urticaria and pruritus, occurred immediately after the first injection, in this way differing from the experimental reaction.

Since this time the appreciation of the condition now known as human hypersensitiveness or allergy has greatly increased and, as we now look back over the literature, we find occasional instances reported of peculiar reactions to many different substances which chemically bear no relation to antitoxic serum. Indeed, a great many reactions, such as those of infants and children to milk, egg, and animal danders, were well known to the laity but occupied no place in medical literature. Peculiar drug reactions, known as idiosyncrasies, were well recognized, but they were not identified as allergies until 1916, when the writer noticed the relatively large number of such cases occurring among hypersensitive individuals and remarked the similarity of symptoms of drug reactions with those of foreign proteins in specifically hypersensitive persons. From our present vantage point we can easily appreciate the reactions obtained by Blakeley (8) with pollens of grasses, by Dunbar (9) with ragweed pollen and by Cole (1) with buckwheat.

Since the clinical conditions of bronchial asthma, hay fever, urticaria, angio-neurotic edema and the erythemas have been definitely recognized as manifestations of human hypersensitiveness and since it has been demonstrated that diagnoses can be made with the well known cutaneous reaction and that therapeutic effects can be obtained by injection, a great deal of work has been done and the results have been published, but a careful review of this literature since 1915 shows only a few records of the general or constitutional reactions following the use of allergens specifically applied to hypersensitive individuals for the purpose of diagnosis or treatment. Rackemann (10) reported reactions in two cases following therapeutic injectionso fextract of horse dander. Walker (11) mentions but incidentally a general reaction following a therapeutic injection of an extract of flaxseed. Gustenberger and Davis (12) reported a reaction with egg protein, the intradermal method being used for testing. The writer (13) has called attention to the dangers of the constitutional reactions on injection and has noted their frequency with pollen extracts as being 3.75 per cent in 4192 injections in 339 hay fever cases up to January, 1916. The occurrence of many other such reactions is personally known to the writer but they have not been reported in the literature.

SYMPTOMS OF THE CONSTITUTIONAL REACTION

The symptoms of general reactions in allergic individuals are entirely distinct and apart from the symptoms that occur in the normal man, even though the latter be given one hundred thousand times the amount of allergen (for example, horse serum). Allergic symptoms are as characteristic as those of a typical lobar pneumonia. The onset of constitutional symptoms may be immediate or they may be delayed up to five days. Discussion of this point is made in greater detail in the section on "Varieties of the Reaction." In general, the symptoms are those of the various clinically recognized allergies and in any individual case they are usually those from which the patient suffers, plus certain manifestations in tissues not reached by the allergen under ordinary exposure. Thus case 2185 has asthma,

coryza and urticaria from eating fish, and asthma and coryza from handling fish glue (LePage glue). He showed identical symptoms in the constitutional reactions after a test and after a therapeutic injection of the sterile solution of LePage glue. On the other hand case 2197, who has clinically only hay fever from timothy pollen and never asthma, developed coryza and in addition asthma and glandular swellings in the neck, following the subcutaneous injection of timothy pollen extract. Case 2180 had, in addition to the usual clinical symptoms, nausea and severe headache for a week following the first injection of horse dander extract. We can group constitutional symptoms as those that are usual and those that are infrequent.

Usual symptoms

Coryza. This term is meant to include the ocular symptoms of corneal injection, lachrymation and itching, as well as the nasal symptoms of discharge, sneezing, and edema of the mucous membrane causing obstruction. The term "Allergic Coryza" has been employed by the writer (14) to designate all forms of vasomotor rhinitis that are allergic in nature. Coryza is a particularly common symptom of general reactions and it may occur on the ingestion of allergens as foods and drugs, as well as when these substances are injected for test or treatment. It is more common, however, with substances such as pollens. that are naturally absorbed by inhalation.

Asthma. This is somewhat more common in cases with clinical asthma but it does occur in clinically non-asthmatic cases, as no. 2197 mentioned above. Asthma is a symptom of the bronchial edema which makes the general reaction dangerous and which may be the cause of death from asphyxia.

Urticaria. This is a very usual manifestation as would be indicated by the frequent occurrence of the immediate skin reaction on test. It is prevented from being a usual clinical symptom by the fact that a sufficient amount of allergen is not absorbed through the respiratory mucous membrane and carried by the systemic circulation to the skin.

Erythema. In some cases the skin becomes scarlet without the appearance of urticarial wheals.

Pruritus. This condition usually accompanies the urticaria or erythema but does exist without either. One case not included in the 1920 series regularly (at least six times) had a very marked pruritus ani within fifteen minutes after an injection of timothy pollen extract.

Edema. The angio-neurotic type of edema may occur in any part of the body, and when once developed, it takes two to four days for the condition to subside. In cases of allergic coryza it is most commonly observed in the tissues of the upper and lower lids so that the eyes may be entirely closed. Occasionally the sclera is also involved and the edematous scleral tissue may protrude between the lids. Edema of the glottis has not been identified though it may have occurred in the fatal case cited later in this paper, and edema of the gastro-intestinal tract may explain some of the cases in which abdominal pain is a symptom.

Cough. This may occur independently of asthma though it is often associated with it. It is violent and paroxysmal like the cough of pertussis and seems to be due to laryngeal irritation.

Infrequent symptoms

Glandular enlargement. This is noted twice in the cases included in the protocol, nos. 902 and 2197. It has not been noted by me in more than six cases. While it is noted as part of an immediate reaction in case 2197, it is certainly not itself a noticeable phenomenon in less than three hours and the swellings usually last three days. The submaxillary and cervical glands, especially the anterior chain, are the only ones that have been observed, except in one case not included in the protocol, in which, following one of the injections, the preauricular glands were so enlarged that the condition was at first diagnosed as a parotitis. In this case following the next injection the same glands became swollen and tender and with them the cervical group. When the same reaction occurred a third time after injection, the time interval in all three instances being about twenty-four hours,

there could be no mistaking the relation between injection and glandular enlargement.

Headache. This may be of a mild type, frontal or occipital, and, like the glandular enlargements, does not develop immediately. It may persist, as in one case, no. 2180, for a week. On the other hand, headache may be excruciatingly severe and of a migrainous type. The writer experienced one such reaction in himself following an injection of horse dander extract. The headache developed in ten hours and gave the sensation truly described as "splitting." It lasted six hours. The writer is otherwise not subject to such headache.

Fever. This, together with chilliness, has been complained of in a few cases but it has not been verified by actual readings except in the case with preauricular adenitis, when a temperature of 101°F. was observed with each of the three glandular reactions. In this case it began in six hours and lasted for twenty-four hours.

Nausea. Nausea sometimes accompanied by syncope and vomiting is not very usual. With violent immediate reactions of the usual type, vomiting may occur inside of an hour and under these conditions it is so copious that there must be an extraordinarily large secretion from the gastric mucosa to account for the volume. In one case, not included in the protocol, in which a constitutional reaction with asthma took place after aspirin by ingestion, the vomiting followed in twelve hours and with it the attack ceased. This was also the usual clinical course, according to the patient, whose attacks had for some time followed the use of aspirin taken for headache on the advice of his physician.

Diarrhea. No cases in the 1920 series exhibited this symptom. I can recall two cases in which diarrhea followed the injection of ragweed pollen extract. The attacks started in six hours and lasted from twelve to twenty-four hours. The regularity of recurrence after injection in both cases is the sole reason for considering it a general reaction. There were no other constitutional symptoms in these two cases.

Acute abdominal pain. This symptom has been noted in a few cases not in this series and has been attributed to an angioneurotic edema of the gastrointestinal tract. Acute abdominal pain, cramplike in nature, over the lower abdomen, developed within one hour after the first ragweed injection in case 2227. The case was not observed during this attack and there is no record of its duration. It was not associated with vomiting or diarrhea. This may have been a type of reaction similar to those discussed under dysmenorrhea. The menses were absent in this case on account of the fact that this patient, a girl, was barely thirteen years of age and the menses had not yet been established.

Dysmenorrhea, or rather an untimely and scanty menstrual flow, following acute cramplike pains in the lower abdomen is recalled in the case of two women aged twenty-six and thirty-eight, respectively. In both of these cases the symptom was part of an immediate reaction with asthma, coryza and urticaria, the menstrual flow itself not being apparent until three hours later and lasting only one day. Among many pregnant women tested and treated no such symptom has ever appeared, but extreme caution is always taken in such cases, for abortion might be induced.

Syncope. This was not noted in any of the 1920 series. It does occur as an immediate effect, namely within one hour, and is usually associated with nausea and vomiting. I recall but one case in which it occurred alone. Reference is not made here to those occasional cases of syncope due to mental or nervous instability in which the patient will give a history of similar attacks following the sight of a hypodermic needle or a drop of blood.

Cardiac collapse. I recall but one case in my entire experience in which this condition took place as a primary symptom. Of course cardiac dilatation and vasomotor collapse do occur secondarily in the fulminating types of reactions with extreme dyspnea and partial asphyxia. But in the case referred to above, there developed, within one-half hour and with an absence of all usual general symptoms, a profound weakness and prostration, pallor and sweating without the loss of consciousness of syncope.

The heart rate was 140 and the pulse was imperceptible, with cold skin and marked sweating. One milligram of strophanthin was given intramuscularly with adrenalin chloride 15 minums. Except for the adrenalin effect the condition was normal within one hour.

From such a categorical list of the symptoms of a general reaction, one draws but a hazy idea of the pictures of the actual reaction in the individual case, but, as case histories will be cited later illustrating the various points to be brought out, no attempt will be made here to fill this deficiency.

VARIETIES OF THE CONSTITUTIONAL REACTION

The constitutional reaction can conveniently be considered as occurring in two forms, the immediate and the delayed.

1. The immediate general reaction

The immediate reaction occurs within one hour after the allergen is introduced. This more or less arbitrary time limit has been adopted by the writer because in practical clinical work one sees a very large group whose reactions fall well within this limit of time, in fact well within one-half hour. In some cases it ensues on the instant (within one minute) and the severity of the symptom is in direct proportion to the brevity in time of onset. The sooner the symptoms begin, the greater their intensity and the greater the danger of a fatal result. On the other hand if cases do not react within the hour, the reactions are usually delayed for a period of at least six hours, and may not make their appearance for five days. No definite reactions have been identified after a longer interval though there seems to be no reason why they should not so occur. The protocol of the constitutional reactions accompanying this paper shows only 3 cases, nos. 2364, 2381, 2473, with 4 reactions, in which the reaction appeared after one hour and under six hours, out of a total of twenty cases with 44 delayed reactions; i.e., after six hours. While this differentiation of immediate and delayed reactions is, for the present at least, based upon confessedly arbitrary grounds, it

serves a definite clinical purpose in that it separates the intense and dangerous reactions from those that are merely subjectively disagreeable.

Allergens may cause immediate constitutional reactions by whatever path they may be introduced systemically; that is, after test, after injection or on ingestion. Since these immediate general reactions may occur when the cutaneous reaction is negative as well as when positive, it will be well to discuss separately the general reactions occurring with allergens which give a positive skin test and those occurring with allergens which give a negative skin test.

a. When the cutaneous test is immediately positive. The immediately and genuinely positive intradermal test is pathognomonic of a cutaneous hypersensitiveness and indicates an accompanying clinical hypersensitiveness of the mucous membranes of the respiratory tract in approximately 95 per cent of the cases reacting by test to pollens and animal epithelia. In other words, the intradermal test reactions agree with the clinical histories or can be clinically substantiated in 95 per cent of the cases reacting to the pollens and animal epithelia. This has been well shown by Vander Veer in the following table based on studies of cases in our clinic.

	POSITIVE REACTION WITH POSITIVE HISTORY	NEGATIVE REACTION WITH NEGATIVE HISTORY	NEGATIVE REACTION WITH POSITIVE HISTORY	POSITIVE REACTION WITH NEGATIVE HISTORY	REACTION AND HISTORY CORRESPONDING
					per cent
Pollens	155	29	4	4	96
Animal epithelium	20	17	2	0	95

When 0.01 cc. of extract is injected intradermally for the test according to the usual technic, a general reaction may follow within a few minutes. Ten such cases are cited in the protocol. One had two reactions with different allergens. Thirty-one cases in the protocol gave 42 immediate general reactions on therapeutic injection of the allergen. This is due directly to an overdose and the time of onset and symptoms are exactly similar to reactions from the test. Following is the history of the case in which death resulted from the test.

Case 2673. Boy, three years of age, developed an attack of asthma at the age of eighteen months. The attack started with cough, then dyspnea and vomiting followed. There was no coryza. The dyspnea became increasingly severe until he passed into collapse and a semi-conscious state with a pulse rate of 160 and marked cyanosis. From this time on he was constantly asthmatic with exacerbations. These severe attacks lasted from one to seven days and occurred about once a month. As he would vomit immediately after ingestion of egg, no eggs had been used for over a year. He had mild urticaria at the age of one year. Eczema had been present for the first two years of life.

Physical examination. The patient was an undersized, poorly-nourished boy. Respiration was labored. He appeared anemic. There was nothing else of note except the sibilant sounds and räles throughout both lungs and a marked double Harrison's groove.

On the first visit he was tested with milk, egg and cereal preparations, eight tests in all. Tests with very dilute egg protein were only suggestive but the casein preparation gave a marked reaction. The cereal preparations were negative. Two days later he was tested with more concentrated egg preparations. Ovomucoid gave a moderate reaction and egg white globulin a marked reaction. The meat extracts were negative. The next day he was tested as follows: (The decimals indicate milligrams of nitrogen per cubic centimeter of solution; 0.01 cc. was used to test.)

Orris root	0.1	negative	Chicken epithelium	0.5	marked
Dust extract		negative	Horse epithelium	0.04	slight
Dog epithelium	0.1	negative	Horse serum	0.1	marked
Cat epithelium	0.1	negative	Rabbit epithelium	0.2	marked

No untoward results had followed these tests. The reactions all subsided within twelve hours. Two days later, November 29, 1920, he was better than usual. The following tests were made:

Tests

Ragweed	0.1		Vanilla
Timothy	0.1		Chocolate
Horse epithelium	0.4		Peanut
LePage glue	0.1	marked	Cocoanut

Within two minutes it was noticed that the reaction at the site of the LePage glue test had spread up and down the arm and there were many

fine urticarial spots appearing all over the arm. The boy then suddenly broke out in a general rash, his face began to bloat with an edema, until his eyes were closed. Cough and dyspnea were marked for a minute, he was deeply cyanotic and respiration ceased, though respiratory efforts continued for a minute longer. Artificial respiration was attempted but no air could be made to enter or leave the chest. The heart continued to beat for a minute after respiration ceased. He had been given 1.0 cc. of adrenalin (1:1000) intravenously at the onset of the attack and strophanthin 0.125 mgm.

In my opinion this was a genuine allergic death from asphyxia and not due to any associated condition of status lymphaticus. I have attributed this death to the LePage (fish glue) solution because the patient was not very sensitive to the horse epithelium as shown by the test on a previous day and because the marked local reaction and urticaria started about the site of the LePage test. In this case the LePage solution used contained 0.1 mgm. of nitrogen per cubic centimeter; 0.01 cc., at most it can be supposed 0.02 cc., was injected. This means that death in this case was caused by a dose containing only 0.001 to 0.002 mgm. of nitrogen.

On ingestion of the allergen in its natural form, where the cutaneous reaction has been positive, a reaction may occur immediately and, if the buccal, esophageal and gastric mucous membranes react as well, either the substance cannot be swallowed at all or it is very quickly vomited. This is the condition in many of the egg allergies of children. Such mucous membrane reactions are in reality local manifestations but in certain of these cases absorption of the allergen into the systemic circulation may take place through the hypersensitive buccal or lingual mucous membrane. In a child of eight years, exquisitely hypersensitive to egg, a mild urticaria developed fifteen minutes after a piece of cake containing egg had been placed against the tongue for a minute. The saliva was not swallowed and the tongue remained protruded until she rinsed her mouth out thoroughly with water at the end of the experiment. The tongue itched and was very red and slightly swollen.

There are cases in which the gastro-intestinal mucous membrane appears not to be sensitive to a substance which may produce a general reaction when the substance is eaten. An example of such an occurrence is presented in the history of case 2826. This individual, a woman of thirty-three years of age, exhibited the symptoms of edema of the lips and face within a few minutes after the ingestion of two hazel nuts and after twenty minutes she experienced a severe attack of asthma that lasted for two hours. She had had such a clinical reaction on two other separate occasions. Urticaria and pruritus had never been a part of this clinical reaction. There were no gastro-intestinal symptoms either at the time or subsequently. Other nuts besides hazel nuts could be eaten with impunity.

b. When the cutaneous test is negative. Occasionally, even when the skin test is negative, a general immediate reaction ensues after cutaneous test, after subcutaneous injection, or after ingestion, that is unmistakable as to its cause, as will be shown in the case 1766 to be cited. This can be explained on the assumption of a complete absence of skin allergy with a hypersensitiveness limited to the respiratory mucous membrane. In my experience this has occurred only with the drugs and particularly with aspirin. The clinical history bears out the above assumption, for in the cases of aspirin allergy, if urticaria has been one of the clinical symptoms a positive cutaneous reaction is obtained but otherwise the cutaneous reaction is negative. The only difference between the general reaction that occurs after ingestion of aspirin and that following its injection is that twenty to thirty minutes elapse before the onset of symptoms when the drug is ingested and only two to five minutes when it is given by injection. This seems to be merely a matter of rapidity of absorption. In all the aspirin allergies observed, with or without positive skin test, there has been no immediate reaction of the alimentary mucous membrane when the drug is ingested but the symptoms of coryza and asthma are the same as when the drug is injected. Vomiting has occurred in several cases eight to twelve hours after ingestion and this act usually terminates the attack. This indicates a central effect. The following case

illustrates the general reaction occurring on test, on injection and ingestion of the drug where the cutaneous test itself was negative.

Case 1766. Age forty, female. This individual has had three attacks of asthma in the last three months but each one has been extremely severe. The first attack started at 3 p.m. She had been in usual health and was on the street at the time and remembers being just able to crawl to her room. She described the sensation of strangling as though a rope were being pulled tight about her neck. She evidently became unconscious for she found herself on the floor at 1:00 a.m. She did not recover for two weeks during which time her chest was sore, as though she had been beaten. There was cough and nausea for several days, no headache, no fever and *no urticaria.* In the next attack eight weeks later she was seen by a physician within one-half hour after the onset of the attack. Adrenalin was given and she was taken to a hospital. The symptoms were the same as in the first attack. The attack was controlled and lasted altogether three-quarters of an hour but she was ill for two days with weakness and prostration. The third attack, exactly similar, occurred four weeks later. It started at 10 p.m., and had lasted for three-quarters of an hour when morphine was given. This attack incapacitated her for three days. Each of these attacks had occurred on the third or fourth day of the menstrual period. Physical examination was practically negative. Closer questioning then revealed the fact that she had been given a prescription for menstrual headaches and that she had taken one of these capsules twenty to thirty minutes before each of the three attacks of asthma. They contained strychnin, quinin and aspirin. Quinin bisulphate and aspirin in solution were injected intradermally. The amount introduced was less than $\frac{1}{100}$ grain of each. At the site of test there was absolutely no reaction, either immediate or delayed. Within fifteen minutes after the test the patient began to cough violently, complained of a sensation of filling of the throat and thickness of the tongue, numbness of the hands with itching and dryness of the lips and throat. There was no erythema or urticaria. These symptoms disappeared in one-half hour. Six days later a test was again done with $\frac{1}{1000}$ grain of aspirin. There was no reaction at the site of test. A similar but milder constitutional reaction followed immediately. Five days later quinin was tested with negative local and constitutional reaction. Then $\frac{1}{100}$ grain of aspirin was given by subcutaneous injection and the same immediate general reaction ensued in five minutes, with no reaction at the site of injection at the time or later.

2. Delayed constitutional reactions

Under "Immediate Reactions" the reasons were given for the establishment of the time limit of one hour for such reactions. Reactions occurring after this time are considered as delayed.

a. When the cutaneous test is immediately positive. The writer has observed a number of instances in which constitutional symptoms have occurred after an incubation period of one hour or more following a subcutaneous injection of the allergen. In no case was this "delayed" general reaction observed to follow the preliminary test or the first subcutaneous injection but always after a number of injections (usually 8 or 10) had been given.

In the protocol appended to this report there are 20 cases in which 44 delayed general reactions occurred. It will be seen that these delayed reactions were always observed after repeated previous injection had been made.

In striking contrast with these observations are the 10 cases in which constitutional reaction followed the preliminary test. In all of these the general reaction occurred immediately.

It must not be inferred from these statements that an immediate general reaction cannot follow the later injections. Indeed such an occurrence has been frequently observed. The length of the time interval between the injection and the onset of symptoms in the treated cases seems to depend entirely upon the amount of allergen injected, for a sufficiently large amount of the allergen may at any time produce an immediate reaction. Rackemann's (10) reported cases bear out this observation. In my own series, case 2292 of the protocol had given delayed general reactions as shown by urticaria, thirty-six hours after each injection from the tenth to the twentieth. But with the twentieth dose (change to new extract) an immediate coryza developed. Another interesting phenomenon in connection with delayed constitutional reactions in which the delay is induced by therapeutic subcutaneous injections is that a local reaction at the site of injection may be entirely absent if injections have been given in approximately the same site, but here again a sufficiently large or a sufficiently concentrated injection of the

allergen will suffice to produce an immediate reaction at the injection site, as well as an immediate constitutional reaction. The phenomenon of the delayed general reaction appears to be an expression of the purely relative insensitiveness which the writer in another article (15) in this Journal has chosen to designate by the term "Hyposensitization." In such cases a marked positive immediate cutaneous reaction is always obtainable with sufficiently concentrated extracts. This induced delay of the constitutional reaction in hyposensitive cases may obtain whether the allergen is absorbed from a subcutaneous injection or from an intracutaneous test. It likewise holds when the allergen is absorbed by ingestion as shown by the following history.

Case 1107. Age two years when seen in 1917. The first time egg was ever given, about one year previously, his face, lips and tongue became swollen, almost at once. He choked, coughed and became dyspneic and cyanotic, vomited several times and complained of abdominal pains. The father has early hay fever and the mother attacks of urticaria. On skin test the boy reacted markedly to the proteins of egg white but not to egg yolk. He was given injections of egg proteins. After seven injections he could eat egg in pudding in small amounts without trouble. After ten injections he could take five teaspoonfuls of soft boiled egg without trouble. Four more injections were given in this year and he could then eat one egg every other day without trouble until April, 1919, when he developed cough and hoarseness. Eight more injections were given. After this it was noted that he could eat an egg every other day without symptoms. When he was fed one egg every day he developed a harsh hollow cough by the second day, thus showing the delayed constitutional reaction. When he was put on two eggs a day he developed edema of the face about eight hours later—again a delayed constitutional reaction. All symptoms of cough and edema disappeared forty-eight hours after egg was discontinued.

b. When the cutaneous test is negative. Delayed constitutional reactions occurring to allergens to which cutaneous reactions are negative belong to an entirely different group from the hypersensitive cases under consideration. The discussion of them is therefore reserved for a later paper.

CAUSES OF CONSTITUTIONAL REACTIONS

1. Mode of introduction of the allergen

All the clinical studies in allergy here presented have been carried out by means of the intradermal test and the subcutaneous injection. Resort is never had to intravenous injection. The rate of absorption from the skin and subcutaneous tissue is approximately the same. General reactions from intravenous injection would undoubtedly be more immediate and more severe. In making the cutaneous and subcutaneous injection it is perfectly possible that at times the point of the needle should lie in a small vein or lymphatic vessel and undoubtedly this does occur at times and may account for some of the constitutional reactions resulting, but such an occurrence is rare and seems unavoidable and is of no practical account in comparison to the causes to be discussed under 2, 3, and 4.

2. The reactivity of the individual

There are all grades of reactivity varying from those that give very marked positive reactions with the weakest dilutions to those that barely can be said to be positive with the most concentrated extracts. Naturally those reacting violently to the weakest dilutions—with the pollen extracts a concentration of 0.0005 mgm. of nitrogen per cubic centimeter—are most susceptible to the constitutional reactions even when tests and injections are properly carried out. An appreciation of this variation in reactivity is of the utmost practical importance in the therapeutic management of the cases.

3. Activity of the allergens

Great difficulty was experienced in the past by the instability of the extracts, notably the pollen extracts. As will be observed by a glance at the protocol, many of the constitutional reactions followed a change of extract; that is, from one that had been in use a few weeks to a new and freshly prepared one. Even by repeating the tests and by diminishing the dose accordingly it

was not possible always to take sufficient account of the deterioration that had resulted and general reactions ensued. In 1921, with the use of extracts prepared by Coca (16) as given in another article in this issue this great disadvantage has been overcome and we now have preparations that have remained practically stable for over ten months.

4. Concentration and dosage of the extracts

The concentration of the allergen used in the test and the total amount given by injection are of paramount importance. Discussion of these questions is reserved for a later paper.

5. Cumulative effects

a. With the same allergen.

Case 2286, cited in the protocol, reacts clinically and by test to a number of curious and unusual allergens, such as celery, skin of peaches, raspberry and in addition has hay fever and asthma from the pollens of daisy, dandelion and ragweed. On May 6, 1920, she was tested intradermally with extracts of daisy, dandelion, ragweed, celery, timothy, apple, lilac, wistaria. The first four extracts gave marked cutaneous reactions. She was then given by injection daisy and dandelion, 0.001 mgm. of nitrogen of each. There was considerable local reaction for twenty-four hours. No constitutional reaction developed. Two days later the same dose of the same extract of daisy and dandelion out of the same bottle produced a constitutional reaction within one hour with asthma, coryza and urticaria as the symptoms. It is difficult to interpret this reaction on the second injection except on the assumption of a cumulative effect, for on the first visit the patient had actually received more than on the second as the tests were done only a short while before the first injection was given.

b. With different allergens. If a hypersensitive patient is tested with ten to sixteen different extracts and happens to be acutely reactive to four or six of them it is the writer's impression that a general reaction is much more likely to ensue as a result of the sum total effect of all the allergens exerting their influence upon the same reacting mechanism. I have no case that affords

absolute proof of this assumption. The suggestion is offered because it has a definitely practical bearing and will serve as a warning against the carrying out of too many tests at the same time.

6. Allergens which have caused constitutional reactions

A list is here given of all the allergens that, in the writer's experience have definitely caused constitutional reactions, either on test, injection, or ingestion:

LePage	Dog epithelium	Orris
Ovo-mucoid	Dog saliva	Dust extract
Egg albumin and globulin	Flaxseed	Aspirin
Pollens, all kinds	Linseed	Quinine
Horse epithelium	Cottonseed	Ipecac
Horse serum	Mustard	Chicken epithelium (feathers)
Rabbit epithelium	Pepper	Duck epithelium (feathers)
Rabbit serum	Buckwheat	Goose " "
Cat epithelium	Wheat	Nuts (peanut, hazelnut)

Having thus outlined the causes of the general reaction it is obvious that they may be avoided by proper care. Granted than an occasional injection or a test may be accidentally delivered in a venule a serious reaction will not occur if; first, extracts used are sufficiently dilute; second, injections are not repeated too frequently; third, tests, not to exceed six or eight, are made at one time. It is not the purpose of this paper to outline instructions for the use of extracts made according to the directions of Coca in another paper in this issue. The object here is to call attention to the dangers inherent in their use. Specific directions for their use will be given in a subsequent paper.

FREQUENCY OF CONSTITUTIONAL REACTIONS

It may be said that in the series presented general reactions have ensued with great frequency and this is true for the year 1920. But it may be added in extenuation, that these results occurred in a serious attempt to determine the limits of diag-

nostic reactivity and to define the limits of therapeutic dosage. Furthermore, many hitherto unknown allergens have been discovered and in the positive identification of these reactions have occurred. The justification, if such is needed, lies in the fact that the study and correlation of the facts has enhanced our knowledge of the nature of allergy, as well as of the technic of diagnosis and treatment. The following table is based upon our series of 473 consecutive allergic cases studied in 1920. All of the cases giving constitutional reactions are included in the appended protocol.

TREATMENT OF CONSTITUTIONAL REACTIONS

A thorough knowledge of the treatment of the reactions should be had by every one attempting this type of study. First of all, one should quickly recognize the onset of the symptoms usually shown by the extensive urticarial wheals developing about the site of the injection or test or by a beginning erythema or short paroxysmal cough or increasing dyspnea. At once a tourniquet should be tightly applied about the arm above the site of the tests or injections in order to prevent the transportation of more allergen to hypersensitive tissue through the systemic circulation by means of the lymph or blood stream. Adrenalin 1:1000, 1 cc. in adults, in children 0.4 to 0.6 cc., should be given at once subcutaneously or, if the reaction is severe, an intravenous injection of the same amount. If there is a continued increase of symptoms the dose should be repeated in two to five minutes. The writer has never seen any ill effects from these large doses of adrenalin and is confident that if it is used often enough and in large enough dose, the serious results of a reaction can be avoided. Adrenalin is the very best drug available. In the presence of a cardiac dilatation from violent respiratory effort or in the presence of vasomotor collapse, strophanthin, 1 mgm., intravenously, (in children, a proportionate dose) should be given without delay. When the attack is controlled and has passed its peak of severity, morphin in proper doses may be used. The writer has never seen any advantage in the use of atropine in these attacks and the use of this drug militates against the best effect of strophanthin, should it be required.

REFERENCES

(1) Smith, Henry Lee: Arch. Int. Med., 1909, **3**, 350.
(2) Brown, Aaron: Jour. of Immunol., 1922, **7**, 97.
(3) Larsen, H., Paddock, R., Alexander, H. L.: Jour. Immunol., 1922, **7**, 81.
(4) Gottstein: Therap. Monatsch., 1896, **10**, 269.
(5) Rosenau, M. J., and Anderson, J. F.: Hygienic Lab. Bull., No. 29, Apr., 1906, p. 79.
(6) Gillette, H. F.: N. Y. State Med. Jour., 1909, **9**, 373.
(7) Park, Wm. H.: Trans. Assoc. Amer. Phys., 1913, **28**, 95.
(8) Blakeley, C. H.: Exp. researches on the cause and nature of hay fever, London, 1873; New observations on hay fever, London, 1889; British Med. Journal, 1898, **1**, 867.
(9) Dunbar: Berl. klin. Woch., nos. 26, 28, 30, 1905; Zeitsch. f. Immuntaetsforsch, 1907, **7**; Deutsch Med. Woch., 1911, **37**, 578.
(10) Rackemann, Francis M.: Jour. Amer. Med. Assoc., 1917, **69**, 889.
(11) Walker, I. Chandler: Jour. Med. Research, 1917, **36**, 459.
(12) Gertstenberger, H. J., and Davis, J. H.: Jour. Amer. Med. Assoc., 1921, **76**, 721.
(13) Cooke, R. A., and Vander Veer, Albert, Jr.: Jour. Immunol. 1916, **1**, 231.
(14) Cooke, R. A.: Tice's Practice of Med., Vol. 5, Page 481.
(15) Cooke, R. A.: Jour. Immunol., 1922, **7**, 219.
(16) Coca, A. F.: Jour. Immunol. 1922, **7**, 163.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

IV. NEW ETIOLOGIC FACTORS IN BRONCHIAL ASTHMA

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The cutaneous test is accepted today as a diagnostic procedure in the study of human hypersensitiveness. The marked immediate cutaneous reaction that can be confirmed repeatedly is the evidence of the hypersensitiveness of the skin. In the writer's article "On Constitutional Reactions," in this issue of the Journal (page 000), it is shown by a table of cases that the typical cutaneous reaction is likewise indicative of clinical hypersensitiveness in at least 95 per cent of the cases reacting to the extracts of such airborne substances as pollens, powdered root of orris and animal danders.

Bronchial asthma was early recognized as the chief clinical manifestation of the hypersensitive state in human beings and many of the cases were readily diagnosed by testing with extracts of such substances as those mentioned above, easily conceived and long known clinically to be important excitants of an attack.

For those cases not thus easily diagnosed an explanation was sought along two lines, first that it was due to bacterial proteins acting as allergens and secondly that the paroxysm of asthma was a reflex effect.

1. BACTERIAL PROTEINS AS ALLERGENS

The thesis has been advanced that the asthmatic paroxysm can be induced by the absorption of bacterial proteins which, acting as allergens, produce the symptoms just as any other

foreign proteins in the specifically hypersensitive man. This was perhaps a natural corollary to the work already done on foreign proteins in hypersensitive humans by Noon, Schloss, Cooke, Goodale, Longcope, Talbot and others.

Since Walker is largely responsible for the term "Bacterial Sensitization" or "Bacterial Asthma," meaning thereby that the bacterial protein, acting as an allergen, is a basic etiologic factor in one of the clinical allergies as bronchial asthma, let me review his work for the facts by which he concludes the thesis has been sustained. He began with a study (1) of the bacterial flora of the nasal and bronchial secretions of asthmatics and recovered principally *Staphylococcus albus* and *aureus, Micrococcus tetragenous* and *catarrhalis* and a new diphtheroid organism. He carried out agglutination tests with *Staphylococcus aureus,* using the serum of 80 asthmatics. In only 3 cases was the test carried out with the serum of the individual from whom the organism was obtained. In 2 of these cases there was agglutination and in 1 there was not. Out of the 80 sera tested, 54 agglutinated, some in dilutions of 1:50 or less and some 1:100 or higher up to 250. No mention is made of any control with the serum of non-asthmatic individuals. He concludes (page 379) that no inferences can be drawn as to the relations between the isolation of *Staphylococcus pyogenes aureus* from the sputum and nasal secretions of patients and the agglutination tests of the sera of those patients.

In his Study III (2) on the sensitization of patients with bronchial asthma to the bacterial proteins, as demonstrated by the cutaneous reactions, he reports on 100 asthmatics. He used a bacterial powder obtained by centrifuging the bacteria from normal saline in which they were washed. They were then washed twice in alcohol, afterward in ether and then pulverized. The powder was put on a scratch and dissolved in $\frac{N}{10}$ sodium hydrate. He observed five types of reaction, three of which he calls positive. One of these positive types is the urticarial wheal which we recognize as characteristic of the positive allergic cutaneous reaction, the second a small papule with surrounding erythema, the other consists solely of hyperemia. These reac-

tions develop in one-half hour at which time the reading is made. There is no reason given for considering them positive except that 67 cases in the group were negative, that is did not give the same hyperemia. Ninety-seven cases were tested with *Staphylococcus aureus* and 19 were considered to be positive. Nine of the 19 gave an urticarial wheal as the evidence of a reaction. There were no controls on non-asthmatic cases reported. His conclusion is that "In patients with bronchial asthma, positive reactions with the protein of *Staphylococcus aureus* are more common than with the protein from other bacteria."

In the next study (3) dealing with the subject Walker turns to "complement fixation and precipitin reactions with the serum of bronchial asthmatics who are sensitive to the proteins of wheat . . . . and bacteria, using these proteins as antigens, and the cutaneous reaction as an indication of sensitization." Six cases of asthma are presented which gave a cutaneous reaction to *Staphylococcus aureus* that was called positive. Three of them showed a positive complement fixation and 3 were negative; but 2 of the 3 negative cases gave positive precipitin tests, while the third case, designated as M. S. was negative. Speaking of this case, Walker says: "Since M. S. was relieved of asthma by *Staphylococcus pyogenes aureus* vaccine, and the case, J. H. N., was greatly improved during treatment with desensitizing doses of *S. pyogenes aureus* protein, the asthma in these 2 cases would seem to be caused by staphylococcus pyogenes aureus." The organism is not reported as having been sought in the nasal or bronchial secretions of this case M. S. In conclusion, Walker states (page 265) that it is not possible to correlate the results obtained with complement fixation, specific precipitation and cutaneous tests. But he then asserts in Study XIII (4) that "the cutaneous reaction has proven to be of great value in determining the cause of asthma from bacterial protein as from other proteins." He then proceeds to a discussion of "the relationship between cutaneous reactions, serum agglutination tests, and bacterial examination of the sputum and nasal secretions in determining the part *Staphylococcus pyogenes*

aureus and *albus* may play in the cause of bronchial asthma." The author states that 30 cases form the chief basis of this paper, whereas only 21 appear accounted for in the text, 5 under protocol I, 2 under protocol II, 5 under protocol III, 3 under protocol IV, used as controls, and 6 other cases without protocols. In conclusion he says: "The cutaneous test has proven to be the safest and best test for determining the bacterial cause of bronchial asthma."

Let us examine these cases and see what constitutes the proof. We must eliminate the 3 control cases, leaving 18 for consideration, 13 of which gave a positive cutaneous reaction to *Staphylococcus aureus.* In only 4 of the 13 cases was the organism recovered. Nine of the 13 were treated with *Staphylococcus aureus* vaccine and the asthma relieved at least temporarily, but in only 3 of these 9 was the organism recovered. In contrast to this there are 5 cases with a negative cutaneous reaction in 3 of which the organism was recovered and all did just as well under treatment with *aureus* vaccine. On the other hand, 12 of these 18 cases gave a positive agglutination test and the organism was recovered in 7 of the 12. Eleven of the 12 were treated with aureus vaccine and relief of asthma was obtained in 10 of the 11 cases. In other words, in would be very difficult for any one to attempt on the basis of such figures to maintain that the cutaneous reaction was any more efficient than the agglutination test. From this point on all of Walker's papers are based on his belief that he has proven the cutaneous reaction in bronchial asthmatics to be as etiologically diagnostic with bacterial proteins as with other proteins, such as egg, wheat, pollens and animal danders.

This critical review of Walker's studies can lead only to the conclusion that he has not brought forth any proof to show that his so-called positive reaction with bacterial proteins, as used by him in asthmatics, has any bearing upon the bronchial condition or is etiologically diagnostic in any individual case cited. He has only shown that asthmatics treated with a vaccine were relieved, irrespective of the reaction obtained with bacterial protein. His published results must be due to what may be

considered as a non-specific effect obtained by vaccine, thus differentiating the bacterial proteins very sharply from the other foreign proteins concerned in allergy with which non-specific results are not obtained.

Rackemann (5) has supported this thesis of intrinsic bacterial asthmas, but he finds the *non-hemolytic Streptococcus* more prevalent, having isolated this organism in 60 per cent of 40 cases. He used the carbolized bacterial suspension for the intradermal test and he describes positive early reactions occurring within a half hour and characterized by the typical allergic urticarial wheal as well as a late twenty-four-hour reaction with redness, swelling and tenderness. Twenty out of 39 cases tested with autogenous vaccine gave a positive cutaneous reaction, both early and late reactions being used as criteria. In a group of 56 cases he obtained 60.7 per cent of positive reactions, which is considerably higher than Walker's figures of 15.7 per cent, but Rackemann's tests were made by the intradermal method and his cases were selected as probably infective in type. Walker's tests were made by the scratch method and his cases were part of a general group of unselected asthmatics. Rackemann states that "Treatment was successful in fairly close accordance with the presence of a positive skin test."

In this work also the therapeutic results are used as a criterion by which this writer concludes that the cutaneous reaction demonstrates the importance of the bacteria in certain types of asthma. The importance of Rackemann's work lies in the fact that in a majority of his cases he demonstrated the presence of the specific organism in the particular individual. We cannot compare the figures of Rackemann, that is 60.7 per cent of 56 selected cases considered as bacterial asthmas on the basis of a skin test, with his later study (6). These later figures show only 108 cases classed as bacterial asthma in a total of 590 cases, that is 18.3 per cent. In this later work the method of diagnosis of bacterial asthma, that is whether by skin test or not, is not stated.

Is there any way in which this question of the bacterial asthmas can be settled? Two years ago the writer (7) stipulated two

postulates which must be fulfilled in order to establish the proof of the causal relationship between the allergen and the clinical reaction and they hold for bacterial as well as for other substances acting specifically upon hypersensitive man.

1. Hypersensitiveness must be demonstrated by one of the following procedures:
    a. A typical local reaction either cutaneous or ophthalmic must be elicited or
    b. The original allergic manifestation must be reproduced at will on the introduction of the substance, either inhaled, ingested or subcutaneously injected.

2. It must be shown that the individual has come in contact in some way with the suspected substance in order to permit it to act an an etiologic factor.

The writer has studied this subject somewhat from the point of view of these postulates. First, bacterial powders were made of various organisms by the alcohol-ether method described by Walker. The powder was then dissolved in carbolized saline to the point of saturation, filtered clear, and used for intradermal test. The organisms included *Pneumococcus*, types 1, 2, and 3, *Staphylococcus albus* and *aureus*, the latter two each made from at least a dozen strains. Tests were made in a series of fifty cases of bronchial asthma and only two, Case Nos. 2783 and 2879, reacted with a typical immediate urticarial wheal, these reactions being confirmed. The first of these two reacted to the extract of *Staphylococcus albus* and *Pneumococcus* type 1, and the second to *Staphylococcus albus*. Cultures of the nasal and bronchial secretions of the two yielded only a *Streptococcus viridans* in the first case and *Streptococcus viridans* and *Micrococcus catarrhalis* in the second. The *Staphylococcus albus* and *Pneumococcus* type 1 were sought but could not be recovered in either case. The cases in which *Pneumococcus* of the fixed types has been recovered in the sputum have all been tested but never yet shown a positive skin test. There was no suggestion that the bacterial extracts would operate as the other known allergens.

Secondly, it is always possible with extracts of pollens, danders, foods, and drugs, to elicit an immediate constitutional reaction

in a hypersensitive individual by the subcutaneous injection, or, in the case of food and drugs, by the ingestion of the specific allergen in sufficient dosage. With the bacterial preparations, however, no similar immediate allergic reactions have been obtained in my clinic and I find no record of such in the literature. The mere increase in the degree of an existing asthma twelve to twenty-four hours later will not suffice as proof.

We have tried by such measures to prove the possible allergic nature of the bacterial reactions and have failed. Further work might be done and certainly better extracts might be prepared, but from such work we were forced to conclude that the bacteria if they operate as fundamental causative factors in bronchial asthma, do not act as allergens *per se*, but in some way not understood today, and that the cutaneous test with bacterial proteins is of no value in the diagnosis of allergy.

In other words, the conception of a bacterial asthma has been based solely upon analogy and the analogy is not upheld by proof. "Bacterial Asthma" has become a convenient term by which to designate many of those cases not reacting to the genuine allergens, but such cases should be classed as undiagnosed even though resort be had to vaccine therapy with apparently good results.

Since the diagnosis of bacterial asthma is not as yet susceptible to positive proof and is only arrived at by exclusion the writer has taken the attitude that it is wiser to continue to search for new factors which can be shown to be specific agents in accordance with the postulates laid down. In this way by increasing the percentage of diagnosed allergic cases the possible cases of bacterial sensitiveness are more accurately separated from the whole group and will lend themselves to more productive study.

2. REFLEX ASTHMA

Most of the present day writers, discussing vasomotor rhinitis and asthma either together or separately, appear to believe that a vasomotor edema of the respiratory mucus membrane with a resulting rhinitis or asthma can be induced by some irritant

acting reflexly upon the membrane. Walker (8) discussing this point says "The causes of symptoms may be classified as mechanical, chemical, odorific and thermal. Among the mechanical causes any kind of dust is the most frequent cause, more especially sweeping dust and hay dust." He further says, "Some of these patients are sensitive to some type of protein which may have rendered their nasal mucus membranes sensitive to these irritants, others are not sensitive to the proteins." This is in part an adoption of Goodale's idea regarding what he has termed olfactory vasomotor rhinitis or pseudohay fever. Goodale (9, 10) mentions the fragrance of certain plants such as lily of the valley, lilac and hyacinth, as excitants of attacks of sneezing as well as asthma, in cases where the tests with the pollens themselves were negative. In other words both these writers conceive that nonspecific irritants acting through a reflex mechanism may be fundamental causes of the asthmatic paroxysm in individuals who are not hypersensitive. This is a return to the idea so well summed up by Osler in the first edition (1892) of his Practice of Medicine. He says

> Briefly stated, then, bronchial asthma is a neurotic affection characterized by hyperemia and turgescence of the mucosa of the smaller bronchial tubes and a peculiar exudate of mucin. The attacks may be due to direct irritation of the bronchial mucosa or may be induced reflexly by irritation of the nasal mucosa and, indirectly, too, by reflex influences from stomach, intestines, or genital organs.

The writer (11) has contended that non-specific irritants can only operate in those cases that are specifically hypersensitive. They are never fundamental etiologic factors. To be sure these non-specific irritants do produce attacks of asthma in cases that have not been diagnosed but certainly in no greater number than in the diagnosed group. The further proof of the contention lies in the fact that these non-specific and reflexly acting irritants cease to operate and to be productive of attacks in diagnosed cases where the paroxysms have been absent for a period of time either from removal of the specific cause or after improvement and relief as a result of specific therapy.

It is the special object of this paper to show that the two supposedly reflexly acting mechanical excitants particularly selected by Walker, namely, hay dust and house dust, are genuinely specific factors and that they operate in the specifically hypersensitive individual just as do pollens, animal dander and the other well known allergens that demonstrate their clinical effect after absorption by inhalation.

A. Hay dust

The first case in which hay dust was shown to act specifically is here described.

Case 2207. A man, forty-five years of age, was seen in March, 1920, when he complained of bronchial asthma. His father was one of three asthmatic children, in a family of ten children. The patient's first attack of asthma occurred in 1900 and came on when he was visiting in a farm house to which a barn was attached. The next attack occurred one year later after he lay down in timothy hay. Subsequent attacks have come on following the handling of objects that had been packed in timothy hay. The patient states very positively that he has no trouble (either hay fever or asthma) in the country or elsewhere during the months of May, June and July, when the grasses are in flower. Tests carried out with the strongest extract of timothy pollen resulted negatively. Tests carried out later in both the skin and the eye with an extract of the timothy hay itself resulted in both instances in definite positive reactions. Similar tests made with the same extract upon normal individuals resulted negatively.

Further use of the extract of the timothy hay has verified the specificity of the reactions. Three of the 327 cases of asthma under consideration were found to be hypersensitive to the substance. These cases indicate that attacks of asthma on exposure to dusty hay are not to be considered as a reflex effect of a non-specific excitant, as Walker states, but as an expression of a specific allergic reaction.

B. House dust

Following is the history of the case that led to the discovery of the presence in house dust of a specific allergen.

Case 1763, T. F., male, age twenty-six, had had frequent attacks of asthma for fourteen years. He then enlisted in the army in June, 1917, and while stationed in Texas had no trouble at all. He returned home in October for six days and had severe asthma all that time. On his return to Texas the attacks disappeared and he was again free until he returned home in December. He applied for treatment in January, 1918, having been continuously ill and unable to work for a month. He was tested by the intradermal method with extracts of all our then known products including pollens, sachet powders, animal danders, foods, and drugs with negative results. He was advised to use an army cot and air pillow. The attacks were lessened. Several times he slept away from home and was well, but the attacks returned regularly when he stayed at home. Finding all tests negative, a culture of the sputum had been made and an influenza bacillus and *Streptococcus viridans* had been isolated. A vaccine[1] had been made and the patient had been treated for over two months with vaccine injections with no improvement in his condition whatsoever. In April of that year, he was instructed to bring all the dust that could be collected from his room, going over it carefully with a vacuum cleaner. This dust was then extracted just as pollens, orris and other substances are extracted, made sterile by filtration and used for intradermal tests. For the first time in this case very marked positive reactions were obtained in the skin at two sites of test and this was followed in a few moments by the development of a mild constitutional reaction with asthma, coryza, general erythema, and pruritus as the symptoms. With proper dilution of the extract this local reaction could always be elicited.

A number of other cases hitherto undiagnosed gave marked cutaneous reactions with this same dust extract injected intradermally. The patient's home was then investigated. It was a clean, modern house. His mattress was made of long curled horse hair, the pillows were feather; there was only a small rug on the floor. He had been negative to extracts of feathers and to horse hair and continued so on repeated testing. Although it was not possible to discover the exact substance giving the reaction, the presence of a specific airborne factor was demonstrated. The patient then moved to California where he has been entirely free from symptoms.

[1] The culture and vaccine were made by Professor John Torrey of the Department of Hygiene, Cornell Medical School. The examination was made from the fresh specimen.

We were then face to face with a new procedure in the diagnostic study of asthma and with a possible *new but unknown* substance. Efforts to discover the active ingredient in the dust extracts by comparative tests has so far yielded negative results. Of course, in houses in which there is a dog or a cat, the extracts of the dust agree by test but in lesser degree with extracts of the dander of the respective animals. The same holds true of the agreement by test of the dust extracts of rooms in which orris root powders are used with the orris extract itself, and this is the fact in those houses where rabbit hair pillows and mattresses are used. But dust collected from homes where none of these

TABLE 1
Reactions

CASE NUMBER	DUST NO. 1	ORRIS	RICE	WHEAT	TIMOTHY	RAGWEED	HORSE	CAT	DOG	RABBIT	FEATHER	GLUE
2185	+++	0	---	---	0	+++	0	0	0	---	0	+++
2183	+++	+++	---	---	0	+++	0	0	0	0	+++	0
2170	+++	0	0	0	0	0	0	0	+++	+++	---	---
2169	+++	0	0	0	0	+++	+++	+++	0	0	0	0
2165	+++	0	0	0	0	+++	0	0	0	+++	0	---
2196	+++	0	0	0	0	0	---	0	0	0	+++	0
2189	+++	0	0	0	0	0	---	0	0	0	+++	0

+++ = Marked positive.
0 = Negative.
--- = Test not done.

articles can be found also gives an extract which produces a marked positive cutaneous reaction in certain cases, and is still an unidentified factor. The extract does not agree by test with any other known extract used. Table 1 clearly demonstrates this point, and in all of these cases a single extract known as Dust Extract No. 1 was used and all tests were made by the intracutaneous method. The cases in table 1 were selected from those in which marked dust reactions were obtained, but it will be seen that while other extracts are occasionally positive there is no agreement between them and the dust reactions. Many similar cases could be cited, but the table is abbreviated purposely as greater length would not add to the argument.

The preparation of the dust extract is carried out according to the suggestions found in Coca's article in this issue of the Journal of Immunology on the preparation of extracts. In brief, we can say that the dust collected by means of a vacuum cleaner is treated first with ether to remove all fatty substances, and is then extracted with the standard extracting fluid. The extraction is allowed to continue for two or three days when the solution is filtered off, sterilized by filtration through a Berkefeld and put through all the sterility tests. This extract contains nitrogen, probably, in many chemical combinations so that these extracts cannot be standardized satisfactorily by the nitrogen content and the nitrogen determination has only a relative value.

While the chemical studies of these dust extracts have not been completed and are still under way, it can be stated here that by dialysis a considerable quantity of nitrogenous substance appears in the dialysate, but that the dialysate does not contain the reacting substance. When the dust extract is sealed in sterile tubes and heated to 212°F. for thirty minutes there is a diminution in the activity of the extract, but under these conditions no precipitate forms. When heated to boiling in an open vessel so that the carbon dioxide of the extracting fluid is driven off, some precipitation takes place and the extract loses all power of reactivity in a very short time.

The question that naturally arises is, what is the actual or relative importance of this new extract? It can be at once stated that as a diagnostic procedure the testing of dust extracts is of the utmost importance in demonstrating the presence or absence of environmental substances which act on absorption by inhalation, and whether or not this environmental factor is domiciliary or occupational. The writer has always been impressed by the importance of the respiratory tract as the chief path of absorption in all adult asthmatics, and the following table arranged for comparison illustrates this point by the large percentage of cases in the inhalation group. In table 2 the cases of Rackemann and of Walker (12) are arranged for comparison with 327 of the writer's cases of bronchial asthma studied in 1920. The figures for 1921 will be published shortly. Racke-

mann's figures are those supplied by him in a personal communication and are more recent than the figures appearing in his article (6). All those cases, such as bacterial, not diagnosed by means of a positive cutaneous reaction are placed in the general group of "intrinsic asthma" and classed as undiagnosed. In this respect the cases of all three authors are treated in exactly the same manner. It will be noticed that the percentages of cases occurring from pollen, danders, powders, and foods do not make a total of 100 in any of the three writers' statistics. This is due to the fact that many cases are examples of multiple hypersensitiveness and belong in two, or even three or more, groups and are therefore counted more than once. Consequently, in order

TABLE 2

AUTHOR	NUMBER OF CASES	EXTRINSIC CAUSES												INTRINSIC CAUSES	
		Absorption by inhalation						By inhalation	Absorption by ingestion				Cases diagnosed	Bacterial asthma. Undiagnosed cases	
		Pollens		Animal dander		Vegetable powder			Food		Drugs				
		Number	Per cent	Number	Per cent	Number	Per cent	Per cent	Number	Per cent	Number	Per cent	Per cent	Number	Per cent
Rackemann	590	150	25.0	55	9.0	9	1.5	27.8	19	3.2	0		31.0	408	69.0
Walker	400	92	23.0	78	19.5	0		23.0	68	17.0	0		40.0	241	60.0
Cooke	327	86	26.0	138	42.0	52	16.0	69.8	12	3.6	4	1.2	73.4	87	26.6

to arrive at the percentage of cases due to substances absorbed by inhalation it has been necessary to take the percentage of cases undiagnosed and by subtraction arrive at the percentage of diagnosed cases. From the percentage of diagnosed cases we subtract the percentage of cases of food reactions and so arrive at the figures for the percentage by inhalation. For example: Among the writer's cases 26.6 per cent are undiagnosed; this means that 73.4 per cent were diagnosed. Only 3.6 per cent of the cases are diagnosed as due to foods, and by subtraction this leaves 69.8 per cent as due to substances absorbed by inhalation. In other words, practically 70 per cent of all the writer's cases studied are due to substances that are conveyed in the form of

dust and that act after absorption by the mucous membranes of the respiratory tract. It deserves some comment that the percentage of cases of the diagnosed group are for Rackemann 31, for Walker 40, and for the writer 73.4 per cent. I believe that these differences can be satisfactorily explained on three grounds. First, the number of routine tests made with extracts of airborne substances. The greater the number of tests the greater the improvement will be in the percentage of positive diagnoses; inevitably this must result in a decrease in the percentage of the so-called bacterial and undiagnosed cases. There are a number of known substances to which Rackemann and Walker appear to attach little or no importance, for either they are not mentioned at all, or only casually mentioned in their writings. For example: Scarcely a day passes at our clinic on which we do not diagnose by positive cutaneous reactions several cases that are hypersensitive to the dander of rabbit and goat. The importance of these reactions lies in the fact that we are able in many cases to demonstrate the presence of these substances in the homes of the individuals reacting. The untreated and unsterilized hair of both rabbit and goat, containing a large amount of nitrogenous substance from the attached dander, is used very extensively, not only in New York but in all large centers of population, by families of foreign birth. The Italians import and use goat hair for pillows and mattresses, while the Germans, Slavs, Poles, and Hungarians, and among these especially the Jews, use rabbit hair for the same purpose. Second, Rackemann and Walker both use the so-called scratch test of Schloss, whereas the writer uses the intracutaneous test which Brown (13), in another article in this issue, shows to be by far the more delicate and the more efficient. Third, the writer uses fluid extracts prepared according to the directions published by Coca (14) in this Journal. These extracts, according to Brown (13) give a greater percentage of positive reactions even when the scratch test is used than do the extracts prepared according to the directions of Wodehouse (15) and made commercially available and used by Rackemann. Walker does not state in his publications what extracts were used by him.

In order to show the importance of the dust extract, actually and relatively, a list is given in table 3 which shows all of the individual substances found to be of diagnostic importance in the 327 cases under consideration. In this table is also put down the number of times each one of these extracts was found to react as a causative factor, and the dust extract was important in 33 per cent of the entire group. It is seen that the number of reactions obtained is much larger than the number of cases studied in spite of the fact that 87 were negative to all tests;

TABLE 3

Total number of cases 327

EXTRACT	NUMBER OF CASES REACTING	PER CENT OF CASES	EXTRACT	NUMBER OF CASES REACTING	PER CENT OF CASES
Timothy	12	3.7	Horse epithelium	41	12.8
Ragweed	74	22.0	Cat "	30	9.2
Orris	47	14.4	Dog "	18	5.5
Rice	10	3.0	Rabbit "	18	5.5
Wheat	8	2.7	Cow "	2	0.6
Corn	11	3.4	Aspirin	3	0.9
Buckwheat	11	3.4	Ipecac	1	0.3
Cottonseed	2	0.6	Egg	7	2.1
Glue	4	1.2	Milk	2	0.6
Hay	3	0.9	Celery	2	0.6
Dust No. 1	109	33.0	Peach	1	0.3
Feather	84	25.7	Raspberry	1	0.3

and this is because a very large percentage are examples of multiple hypersensitiveness. What has been said in this paper for the importance of dust extracts in asthma obtains in the same way and in the same degree in allergic coryza.

CONCLUSIONS

In this paper it has been shown that the group of substances absorbed by inhalation play a much more important part as specific causative factors of asthma than is generally considered to be the case by other investigators. Diagnoses arrived at in accordance with this idea may be made with a greater degree of assurance on account of the fact that they are based upon posi-

tive findings and not upon negative findings, as is the case with the so-called bacterial asthma cases, which the writer insists are to be more properly classified as undiagnosed. The new procedure of testing dust extracts has yielded valuable information in that it permits a study of the occupational or domiciliary environment of an asthmatic and establishes a positive diagnosis in certain cases not obtainable by any other means. Further, it has shown the presence of a substance in most house dusts that is in itself an important factor, but the nature and source of which is as yet unknown. The dust of hay, also, may act as a specific allergen and is not to be considered solely as a simple mechanical irritant.

REFERENCES

(1) Walker, I. Chandler: Jour. Med. Research, 1917, **35**, 373.
(2) Walker, I. Chandler: Jour. Med. Research, 1917, **35**, 487.
(3) Walker, I. Chandler: Jour. Med. Research, 1917, **36**, 243.
(4) Walker, I. Chandler: Jour. Med. Research, 1917, **36**, 295.
(5) Rackemann, Francis, M.: Jour. Immunol., 1920, **5**, 373.
(6) Rackemann, Francis, M.: Am. Jour. Med. Sci., 1921, **162**, 807.
(7) Cooke, R. A.: Tice's Practice of Medicine, Vol. 5, Page 496.
(8) Walker, I. Chandler: Jour. A. M. A., 1920, **75**, 782.
(9) Goodale, J. L.: Boston M. & S. Jour., 1916, **175**, 181.
(10) Goodale, J. L.: Boston M. & S. Jour., 1918, **179**, 295.
(11) Cooke, Robert A.: Tice's Practice of Medicine, vol. 5, pages 499 and 513.
(12) Walker, I. Chandler: Boston M. & S. Jour., 1918, **179**, 288.
(13) Brown, Aaron: Jour. Immunol., 1922, **7**, 97.
(14) Coca, Arthur F.: Jour. Immunol., 1922, **7**, 163.
(15) Wodehouse, R. P.: Boston M. & S. Jour., 1916, **175**, 195.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

V. THE PREPARATION OF FLUID EXTRACTS AND SOLUTIONS FOR USE IN THE DIAGNOSIS AND TREATMENT OF THE ALLERGIES WITH NOTES ON THE COLLECTION OF POLLENS

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From the beginning of his work upon the diagnosis and specific treatment of the allergies (hay fever, asthma, the urticarias), Robert A. Cooke (1, 2) has employed for both the diagnostic and therapeutic injections, fluid extracts of the various materials containing the exciting agents of these conditions. These extracts were originally made with physiological salt solution to which was added the usual percentage of carbolic acid The preparations were standardized according to their nitrogen content. Cooke and also Cooke and Vander Veer, and Vander Veer, have reported their successes in the use of these extracts as diagnostic and therapeutic preparations At the time when these investigators became associated with the writer in the organization that has been established in the New York Hospital for the diagnosis and treatment of the allergies, some of the extracts that had been in use were subject to certain disadvantages. In particular, the pollen extracts were often quite unstable in their activity both as diagnostic agents and as therapeutic material; the extract of feathers, especially chicken feathers, seemed to possess little or no specific activity in cases of known hypersusceptibility to these materials.

The preparation of fluid extracts was undertaken by the writer with the purpose of overcoming the disadvantage mentioned. It was necessary to provide a sterile preparation of not too low

concentration which would remain stable for at least six months. The pursuit of this problem was attended with some serious difficulties which impeded its purpose. One of these difficulties lay in the fact that the sole test object was a human being suffering from a distressing condition, which might or might not be relieved by the therapeutic use of the new kind of protein preparation.

There was, therefore, a natural hesitancy to change from one kind of preparation to another for experimental reasons. Furthermore, the comparison of different preparations by means of the cutaneous or of the ophthalmic reaction is interfered with by the natural difficulty of obtaining consent of suitably sensitive individuals to submit to the experimental injections. The problem was further complicated by the number of the possible factors concerned in the deterioration of the preparations; for example, light, temperature, chemical reaction, quality of glass in which the preparations were stored, and sterility.

Indeed, it must be admitted at the outset that some of these factors have not yet been investigated. It is not known why the preparations in present use satisfy the requirements of potency and relative stability; whether on account of mere sterility or on account of a modification of the composition of the extracting fluid. In any case the preparations about to be described are to be looked upon as merely modifications, perhaps only slight ones, of those employed previously by Cooke and his associates.

While the problem is still under investigation it has been found desirable to make the present preliminary publication on account of the thoroughly successful diagnostic and therapeutic use of which the preparations have been found capable.

In deciding upon the composition of the extracting fluid, the fact has been borne in mind that the exciting agents often reach the sensitive mucous membrane in a dry state. Hence it could be assumed that the active principle in these materials was soluble in a slightly alkaline and saline solution. While it was known that certain vegetable proteins for example, gliadin, are soluble in aqueous media only in the presence of free alkali (sodium hydrate in hundredth normal concentration or stronger),

it was believed that such proteins could have little significance in human hypersensitiveness since they are not soluble in the natural secretions of the mucous membranes. Free alkali was thought to be disadvantageous as an extracting agent on account of its known tendency to denature certain proteins. The desired alkalinity was therefore obtained with the use of sodium bicarbonate. This salt has the desirable faculty of neutralizing both free acid and free alkali and is able, in consequence, to correct any change toward either reaction which they tend to develop in the course of preparation, or during storage.

After some experimentation, the following composition of the extracting fluid was adopted.

Sodium chloride.. 0.5 per cent
Sodium bicarbonate....$NaHCO_3$ in such concentration that 10 cc. of the final fluid equalled about 3 cc. of $\frac{N}{10}$ alkali
Carbolic acid.....................In final concentration of 0.4 per cent

The solution was made without the use of heat and with the avoidance of excessive shaking. When it was desired to dilute an extract, or other preparation with this fluid after the original extract had been sterilized, the diluting fluid itself was sterilized by filtration through a sterile Berkefeld filter.

As a general rule this alkaline extracting fluid is used for all dry materials such as the cereals, the danders, the nuts, and the pollens. It is also used for certain vegetables that contain little juice, such as sweet potato, fresh beans, fresh peas and for the meats. The extracting fluid is used as a diluting fluid when dilution of the extract is desired. When the original material contains considerable fluid such as the fruits and most of the vegetables, it is advantageous, in order to avoid too great dilution, to use a "preserving fluid" containing the constituents of the "extracting fluid" in a higher concentration. Such a preserving fluid we have prepared containing 2.5 per cent NaCl, 1.25 per cent $NaHCO_3$,2. per cent carbolic acid. It has been found that many of the fruit juices contain much more than enough acid to neutralize all of the $NaHCO_3$ in the added preserving fluid. We do not know that the resulting partial neutralization of the acid is of any advantage in the preparation of

the acid juices. For several reasons it has not been possible to make an adequate study of the method of preparing these juices. We have used the preserving fluid referred to for both the neutral and the acid juices on the ground of convenience.

The extraction of the dry materials is carried out at room temperature and usually this extraction is continued for forty-eight hours—sometimes for three days. In these conditions the concentrations of carbolic acid (0.4 per cent) in the extracting fluid is not always sufficient to present the multiplication of bacteria in the mixture. This difficulty was met with the use of toluol, which is able not only to prevent bacterial growth but to kill nonspore bearing organisms. When the material to be extracted is in the form of a powder or fine meal it is advantageous to mix the toluol with the powder before adding the extracting fluid as this makes certain that the toluol reaches all of the substance. It also prevents the formation of clumps which are difficult to break up.

If the material contains oily substances which may interfere with the infiltration of the extracting fluid, the oil is first removed with ether which does not denature proteins. We have not found it necessary nor convenient to use a special extraction apparatus such as the familiar Soxhlet apparatus. We have simply mixed the ether with the substance in a sedimenting jar and as soon as sedimentation was complete we have decanted the ether and made further extractions in the same way with additional portions of ether.

As the different materials contain different percentages of oily substances, the number of changes of ether must be varied. With nuts and oily seeds, we have been guided in this respect by making a rough determination of the quantity of oil removed by the successive fractions of ether. This determination was made by evaporating a few cubic centimeters of the etherial extract in a beaker immersed in hot water and noting the quantity of the oily residue. With the pollens the extent of the oil extracted could be judged by the depth of color imparted to the successive portions of ether. Complete extraction of the oily substances has not been found necessary for some materials, such

as the nuts and the pollens. After the extraction of most of the oily substances the ether has been driven off from the material before it was mixed with the extracting fluid.

In most of the extracts and preserved juices a precipitate forms upon standing. As precipitation continues even after the fresh extract has been filtered, it is necessary to wait until the precipitation is complete before carrying out the further steps of the preparation. In the case of some of the vegetables, this precipitation has been found to cause relatively little reduction of the nitrogen content of the extract. On the other hand, the precipitate in the extract of the meats and fish is doubtless wholly proteid. Conceivably some of the exciting agents of the allergy to the original material are in part or entirely lost by this precipitation. We have no evidence to offer on this point. The separation of the precipitate from the fluid extract offers difficulties varying in degree with the different materials. If the precipitate is not too voluminous it can be removed at once with the use of the Sharples centrifuge. This instrument, which is almost indispensable in the preliminary clearing of larger volumes of fluid, is not adapted to the handling of quantities much less than five hundred cubic centimeters. The smaller quantities of extract may be filtered through paper, preliminary to the final sterilizing filtration through Berkefeld or similar filters.

The precipitate that forms in some of the extracts is so voluminous that it is impossible to use the Sharples centrifuge for its removal. In such a case, a partial separation can usually be effected with the use of a fine mesh towel laid over a sieve. As the precipitate tends to form an impervious mat upon the cloth, it is necessary at intervals to scrape off the collected precipitate with a large spoon. During the period in which the sedimentation is taking place in the extract it is advisable to keep the fluid covered with a shallow layer of toluol. To prevent evaporation of the toluol the container should be tightly stoppered. Many of the extracts are found to be sterile after having stood for three days or longer under toluol. Advantage has been taken of this circumstance in the preparation of some protein substances, particularly the isolated globulins, which are not at all

or with difficulty filterable through the Berkefeld type of filter. However, sterilization of the extract has been secured for much the greater part by Berkefeld filtration.

The filtering flasks were sterilized as usual with dry heat. The filters were sterilized by one exposure in the autoclave to steam under a pressure of fifteen pounds for fifteen minutes. The connecting perforated rubber stoppers were boiled for ten minutes in 5 per cent carbolic acid and again for ten minutes in water. The main stock of the filtered extract was stored in sterile 16-ounce bottles. For clinical purposes extracts are distributed in vaccine bottles or homeopathic vials, which are capped with "No-air" stoppers. The greater part of the stopper must be cut off with scissors so that the cap may be more readily punctured by the syringe needle in removing the contents of the bottle for test purposes. The bottles and caps are sterilized separately by boiling for twenty minutes in plain water. The caps are held firmly on the vials with the use of narrow rubber bands.

Sterility tests have been made by introducing about 0.25 cc. of a filtered preparation at the bottom of a 12- to 15-cm. column of neutral nutrient agar containing 1 per cent of dextrose. Another portion of the preparation was deposited at the surface of the agar in the same tube. The agar was quickly solidified by placing the tube in cold water. The nitrogen content of all the preparations was determined by the Kjeldahl method and generally adjusted by dilution to 0.5 mgm. or less per cubic centimeter. Further dilutions of these "stock solutions" were made for diagnostic and therapeutic use.

It is not in the province of this article to discuss the dosage of the different preparations nor the dangers attending their use. These questions are considered in the various publications of Cooke, Vander Veer and Brown. It may be stated here, however, that the nitrogen content of the usual dilutions of all the pollen extracts was: 0.1, 0.05, 0.01, 0.005 and 0.001 mgm. With many of the vegetables and fruits it was thought advantageous to leave the nitrogen content at its original concentration.

While there are some general principles that apply to all of the members in each of the several groups of materials, the method

employed for each group must be modified to meet certain peculiarities of some of the members of the group. It is intended to refer to some of these peculiarities, although it is not possible in the limits of this communication to describe the procedure followed in each of the individual extracts that have been prepared. It will be useful to describe in some detail the method employed in several instances under each group.

Dry materials

Whole wheat flour: One kilogram of the flour was thoroughly mixed with 150 cc. of toluol and then with 2200 cc. of extracting fluid. After twenty-four hours at room temperature in which the mixture was once thoroughly stirred up the supernatant fluid was drawn off into a filtering flask with the use of negative pressure. With the sediment 2000 cc. of fresh extracting fluid were mixed and after a further period of twenty-four hours the supernatant fluid was drawn off as before and mixed with the first extract. Three liters of fluid were thus obtained. A slight sediment settled in the fluid over night, which was not greatly increased in the next few days. The supernatant fluid was filtered through a Berkefeld filter. Filtration was slow, but not so slow as it was with an extract of another sample of whole wheat flour which had been prepared previously. One cubic centimeter of the filtrate was found to contain 175 mgm. of nitrogen.

Rice polish:[1] 1½ pounds of rice meal were mixed with 150 cc. of toluol and this mixture was stirred well first with one liter of extracting fluid; to this mixture was then added a second liter of extracting fluid; the mixture was left at room temperature until the following day when 300 cc. of supernatant fluid were drawn into a filtering flask. 750 cc. of extracting fluid were mixed with the sediment and on the following day an additional 750 cc. of supernatant fluid were obtained and mixed with the first extract. After a first filtration through the Berkefeld filter the nitrogen content per cubic centimeter was 1.4 mgm. A slight

[1] A generous quantity of this material, which is also called rice meal, was kindly donated by the Louisiana State Rice Milling Co., 100 Hudson Street, New York City. It has been assumed that the allergen of rice is a protein which is the same in all parts of the grain. In accordance with this idea the rice preparation has been made from the materials ("rice bran" and "rice meal"), which are removed from the grain in the process of polishing, because these materials contain about 14 per cent of protein.

precipitation made a second filtration necessary. The nitrogen content after this final filtration was practically the same as it was after the previous filtration.

Dry "navy" bean: The dry beans were ground in a meat chopper and the coarse meal was then ground in a coffee mill. The powder was mixed with toluol and extracting fluid. After twenty-four hours the fluid was pressed out through a clean towel and centrifuged in a Sharples centrifuge. The extract was covered with toluol in a tightly stoppered flask and allowed to stand at room temperature. After five days a voluminous precipitate had formed and settled. The precipitate was removed in the Sharples centrifuge. After a further five days under toluol a second precipitate had formed which was likewise removed by centrifugation. Precipitation continued for several months. A clear extract was obtained at the end of six months in which no further precipitation had taken place one month later. One cubic centimeter of extract contained 5 mgm. of nitrogen.

Horse dander: A quantity of dander obtained by currying and containing few hairs was mixed with three volumes of ether by stirring. After the sediment had completely settled the ether was decanted and discarded. The material was again extracted with another equal portion of ether. The ether was completely removed from the sediment by stirring the latter in a beaker which was immersed in hot water (50 to 60°C.). Fifty grams of the resultant material were mixed with 100 cc. of toluol and then with 1000 cc. of extracting fluid. The mixture was allowed to stand over night in a stoppered flask. During this period the mixture was shaken once. On the following morning the entire mixture was shaken up and thrown on a hardened filter paper. Eight hundred cubic centimeters of a clear brown filtrate were obtained after six hours, this quantity being increased to 800 cc. over night. The filtrate was immediately filtered through a Berkefeld filter. One cubic centimeter of the filtrate contained 0.5 mgm. of nitrogen. The filtrate remained clear.

Pollen: 90 grams of ragweed pollen were treated with ether in the same way as was the horse dander until the decanted ether showed only a slight yellow color. Four or five extractions suffice for ragweed pollen. The grass pollens contain less oil than the ragweed pollen. After the ether had been removed from the pollen as in the case of the dander, the entire quantity of pollen was mixed with 2700 cc. of extracting fluid. The mixture was covered with toluol as usual. After four days, during which time the sediment was shaken up once or

twice daily the supernatant fluid was decanted and the sediment was mixed with a second portion of 450 cc. of extracting fluid. As soon as the sediment had settled the supernatant fluid was decanted and mixed with the first portion. The combined decanted fluid, amounting to 3000 cc. was filtered through a Berkefeld filter. One cubic centimeter of filtrate contained 0.3 mgm. of nitrogen. The filtrate remained clear. One cubic centimeter of an extract of timothy pollen made in the same way contained 0.42 mgm. of nitrogen.

Some idea of the rate of deterioration of the pollen extracts was obtained in the following experience:

Four different preparations of the extract of timothy pollen were made with the extracting fluid in the manner above described.

Preparation Ta was made March 4, 1921 and kept in the ice-box.

Preparation Tb was made December 9, 1921.

Preparation Tc was made November 10, 1920, and kept at room temperature (15 to 32°C.).

Preparation Td was made February 12, 1921, and kept at room temperature.

The nitrogen content of the four preparations per cubic centimeter was as follows: Ta—0.4 mgm., Tb—0.36 mgm., Tc—0.28 mgm., and Td—0.336 mgm. All of the older preparations had been preserved in their original concentration. A few days after preparation Tb was made, the four preparations were diluted to an equal nitrogen content in three different concentrations and compared in the clinic as to their specific activity by intradermal injection in an individual subject to early hay fever. The results are shown in table 1.

These results indicate that the pollen extracts deteriorate somewhat in nine months if they are kept in the ice-box and that this deterioration is considerably greater if the extracts are kept at room temperature.

Feathers: Feathers are washed once with enough ether to wet them well. The ether is wrung out by hand and the feathers are spread upon clean newspapers to dry. The removal of the ether is hastened by occasionally turning over the mass of feathers and by compressing

them. The feathers from which most of the oil has been thus removed are passed in successive batches through 2 one-liter portions of extracting fluid, each batch being allowed to remain about ten minutes in each portion of fluid. Naturally the successive batches of feathers carry a certain quantity of fluid with them after the immersion, and this circumstance places a limit on the quantity of feathers that can be treated in this way by the two liters of extracting fluid. On the other hand, the more feathers used the greater will be the concentration of the extractable protein in the resulting extract. After the last batch of feathers has been carried through, the two portions of fluid are combined and cleared in the Sharples centrifuge. As precipitation sometimes takes place in the extracts of feathers it is well to allow them to stand for a few weeks under toluol before the final Berkefeld filtration.

Hair and wool are treated in the same way as feathers.

TABLE 1

Showing the relative specific activity of the different extracts of timothy pollen on intradermal injection

	0.001*	0.005	0.01
Ta	++	+++	++++
Tb	+++	++++	++++
Tc		+±	+++±
Td		++	+++±

* Milligrams of nitrogen per cubic centimeter.

Moist materials

Moist materials, from which little or no juice can be expressed by hand, such as meats, sweet potato, green pea, turnip, cauliflower, and lima bean, are passed twice through a meat chopper, mixed with extracting fluid and covered with a thin layer of toluol. The extraction is generally interrupted after twenty-four hours and the fluid is obtained by pressing the mixture in a stout towel by hand. The amount of extracting fluid used varies with the nitrogen content. For the meat three or four volumes of the fluid (figured on the weight of the material) are used. For the vegetables, one or two volumes.

Some of the shell fish yield a juice in addition to the meat. To the juice one-quarter of its volume of preserving fluid is added. With the chopped meats three or four volumes of extracting fluid are mixed. These two portions are then thrown together and allowed to stand under

toluol for at least two weeks. With some of the fruits and vegetables that can be peeled, such as peach, tomato, orange, lemon, and grape fruit, it is advantageous to make separate preparations of the pulp and the peel. The pulp in such case is carefully separated from the remainder of the fruit and the juice is obtained from it by squeezing through a towel; the peel is ground in a meat chopper and mixed with a quantity (not too great) of extracting fluid. Juices of fruit and of some of the vegetables, such as string bean, celery, cabbage, lettuce, spinach, cucumber, and white potato, are mixed with one-quarter of its volume of preserving fluid and allowed to stand in well stoppered containers under toluol with which the fluid should be once thoroughly shaken.

Miscellaneous preparations

In general, heat has been avoided in the preparation of the extracts, although according to Wodehouse this precaution is not necessary in the case of some of the vegetables. In the preparation of fish glue, the glue has been diluted with ten times its weight of 0.5 per cent sodium chloride. The diluted material has been sterilized by boiling.

Egg preparations

As in the early experiments of Schloss, different proteins of the egg have been isolated for separate testing. The first step in all the methods of separation of the egg white proteins consists in dissolving the egg white in water. This process is expedited by forcing the whites through a moist towel into the diluting fluid. The eggs used for the purpose were strictly fresh (not more than a few days old). Euglobulin was obtained by saturating a 30 per cent solution of egg white with sodium chloride. The globulin was purified by repeated precipitation. The final precipitate was dialyzed against 1 per cent sodium chloride in the presence of toluol. The diluted material was kept under toluol until the solution was found to be sterilized. This method of sterilization was made necessary by the fact that the globulin could not be filtered through the ordinary Berkefeld filter. The use of the preparation was discontinued because no way was found to prevent continued precipitation of the substance.

Pseudoglobulin, crystalline egg albumin and ovo-mucoid were separated in the usual way, except the final stages of precipitating and washing the coagulable proteins with alcohol. This step was omitted because of the resulting denaturing of the proteins. These proteins can be sterilized by filtration.

In carrying out the cutaneous test upon individuals believed to be sensitive to egg white proteins it is convenient to make a preliminary test with the whole egg white. This procedure, however, suffers from the disadvantage that the several component proteins of egg white are not present in equal concentration. At the suggestion of Dr. Cooke we have attempted to overcome this difficulty by mixing equal volumes of solutions of globulin, albumin, and ovo-mucoid. These solutions contain the respective isolated proteins in the same concentration; namely, 1.5 mgm. of nitrogen per cubic centimeter. The globulin had been separated by repeated precipitation with half saturated ammonium sulphate. This preparation no doubt contained albumin. It included both euglobulin and pseudoglobulin. The ammonium sulphate had been removed by dialyzing against changes of 1 per cent sodium chloride. Toluol was used as an antiseptic during the dialysis. The albumin fraction was obtained from the filtrate after the separation of the globulin by the addition of a little acetic acid and by increasing the $(NH_4)_2SO_4$ saturation to a little over 7 per cent. Both crystalline and non-crystalline albumin were thus included in this fraction.

Dilutions of the egg and milk proteins have been made with 0.4 per cent carbolic acid in 1 per cent sodium chloride. This diluting fluid was sterilized by Berkefeld filtration.

Carbolic acid should not be mixed in concentrated solutions, such as 5 per cent, with the diluted egg proteins, as this causes precipitation of some of the proteins.

Yolk proteins: For the separation of ovo-vitellin we have used the method of Levene and Alsberg, up to the point of extraction with alcohol. This latter step was omitted. The final precipitate was dissolved in 10 per cent sodium chloride and mixed with an equal volume of distilled water which was added slowly with constant stirring. Since the substance is insoluble in physiological saline solutions it is possible that even a skin potentially sensitive to it would fail to react to its injection. No diagnostic reactions have been obtained with the preparation. Yolk proteins soluble in physiological salt solution.

The following method has been used to obtain yolk proteins that are soluble in physiological saline solution:

The yolks were separated from the whites and placed in 1 per cent sodium chloride solution. Each yolk was lifted out of the fluid in a large spoon and the chalazae and film of egg white were removed with the use of a soft towel and scissors.

After a further rinsing in a large dish of 1 per cent sodium chloride solution each yolk was again lifted out of the fluid on the spoon and the surface was dried with a towel. The yolks were broken up in a large separatory funnel and treated with several portions of ether, then with about $\frac{2}{3}$ to $\frac{3}{4}$ volume of 10 per cent sodium chloride. To the resulting solution were added 5 to 6 volumes of distilled water which precipitated the ovo-vitellin. The precipitate was removed by paper filtration. The filtrate was acidified with a few drops of 40 per cent acetic acid and mixed with somewhat more than an equal volume of saturated ammonium sulphate. The resulting precipitate was collected on a paper filter, placed in a dialyzing bag (fish skin) and dialyzed against changes of 1 per cent sodium chloride until no trace of sulphate remained in it. Toluol was depended upon for sterilization.

Milk proteins: Casein is precipitated as usual with acetic acid and separated from the fluid by straining through a stout towel. The towel containing the casein is immersed in changes of water in which the casein is thoroughly washed. With the aid of a pestle, the casein is forced through a wire kitchen strainer, while an assistant keeps the casein well moistened with distilled water. To this suspension of the precipitated casein in water 4 per cent sodium hydroxide is slowly added, while the mixture is continually stirred. The addition of the alkali is continued until early all of the casein is dissolved. Stirring is continued some time beyond this point and if some casein still remains undissolved the solution will be neutral to litmus.

The mixture is filtered through paper and the filtrate is kept under toluol. Before filtration through a Berkefeld filter, the solution is further diluted with 0.4 per cent carbolic acid in 1 per cent sodium chloride to a nitrogen content of 2 mgm. per cubic centimeter. After filtration, the nitrogen content per cubic centimeter is further reduced to 1 mgm.

From the original acetic acid filtrate the other proteins are precipitated with the addition of $1\frac{1}{2}$ volumes of saturated ammonium sulphate and the precipitate, after its collection on a paper filter is freed of the ammonium salt by dialysis against changes of 1 per cent sodium chloride.

COLLECTION OF POLLENS

In the collection of pollens, two principal requirements have been laid down. The first of these has been to obtain the pollen as free as possible from other material (dust from the soil and

from other parts of the plant); the second equally important requirement is that all moisture be eliminated from the collected pollen before it is stored. The method of collection is different with the different groups of plants; that for the grasses is as follows: the heads are cut off with scissors and spread, in not too thick layer, upon strips of glazed paper.

In order to obtain the pollen as free from extraneous matter as possible it is important to shake the collected heads vigorously over a sieve or coarse wire netting before spreading them on the paper. The room used for this purpose should be warm, dry, well lighted and protected from dust. The attic rooms of the ordinary house are generally suitable. Ordinarily the greater part of the pollen obtainable in this way drops out of the heads within twenty-four hours. It has been found, however, by transferring the heads to a fresh sheet of paper at the end of twenty-four hours, that an additional quantity of pollen can be obtained in the second twenty-four hours. For this reason alone it seemed profitable to leave the heads on the glazed paper for at least forty-eight hours. A more important advantage, however, is offered by the observance of this longer period. This advantage is the more thorough drying of the pollen which is so necessary to its proper preservation. Pollen that has been stored in tightly stoppered bottles without first being allowed to dry tends after a time to form clumps and may even become united in a single mass, perhaps under the influence of bacterial growth. On the other hand, if it is sufficiently dried, the material remains indefinitely in the form of a fine yellow powder.

If the pollen in this condition is protected from moisture as in a tightly closed fruit jar, the activity of the extracts made from it after at least a year's standing corresponds as in the beginning with the nitrogen content. There is thus no evidence of deterioration.

At the end of forty-eight hours the heads are taken up from the paper and rubbed in a large wire sieve in order to obtain most of the pollen that still adheres to them. The pollen together with the dried flowers of the grass, which likewise have dropped off or are rubbed off in the sieve, are collected in one place by raising

the ends of the paper strips and tapping the under surface of the paper. The whole mass is then emptied into a clean enameled pan. Most of the dried flowers can be removed from this mass by preliminary sifting with a fine domestic sieve of about four inches diameter. The final sifting should be carried out with a 200-mesh copper wire sieve with the use of a soft, fine, hair brush of one to one and a half inch breadth.

The season for collection is different with the different grasses. That for the most important member of this group, the timothy grass, begins in the neighborhood of New York City about June 20 and ends practically July 6. The heads are not ready for picking until some stamens have appeared on them. The most favorable time of day for collection is in the late afternoon and evening. We have an impression that the yield of pollen is somewhat greater when the heads are collected soon after a shower.

The season for collecting ragweed pollen begins about August 20 and ends about September 5. The method used for the grasses is not applicable here. It was thought necessary, therefore, to shake off the pollen as it ripens naturally in the field.

The pollen of a single plant does not ripen all at one time but over a period of a week or longer. Several of the sessile anthers on a single bract open each day and the contained pollen is released. This process takes place usually in the morning soon after the sunlight has reached the plant. It is profitable to begin the collection as soon as the anthers have opened because after this has taken place the pollen begins to drop out by its own weight or is carried away by the wind, which generally begins to blow after sunrise. The pollen is collected by bending the tops of the plants down so that they can be inserted into the collecting bags and gently shaken. The pollen is readily loosened in this way. Too vigorous shaking loosens the pollen on other parts of the same plant. It requires some dexterity to get the tops into the bag before the pollen has been jarred off. The bags are made of the same glazed paper as was used for the collection of the timothy pollen, and they measure about 11 by 11 inches.

While the foregoing method of collecting ragweed pollen will always be useful for small-scale collection, it will be superseded for collection on a large scale by the method about to be described.

After the bracts have begun to open the stems that bear them are gathered and allowed to dry. When the bracts are thoroughly dried they are stripped off the stem and ground in a mortar or in a wooden ball mill in order to loosen the pollen. The ground mass contains not only the pollen but also other portions of the bracts. It was observed that the pollen sediments rapidly in ether but floats in carbon tetrachloride and it was suggested by Olin Deibert of this division that in a mixture of these two fluids the pollen alone might float whereas most of the other parts of the plant might sink. After some experimentation by Deibert a mixture of 75 parts of carbon tetrachloride and 25 parts of ether was found to effect the desired result. The pollen recovered by this method may be washed with two changes of ether, then dried and sifted in a 200-mesh wire sieve and preserved in tightly stoppered bottles.

REFERENCES

(1) Cooke, R. A.: Laryngoscope, 1915, **25**, 108.
(2) Cooke, R. A. and Vander Veer, A.: J. of Immunol., 1916, **1**, 201.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

VI. DERMATITIS VENENATA

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Among the various forms of human hypersensitiveness, the susceptibility to "poison ivy" presents certain peculiarities which make it particularly interesting. One of these peculiarities is the constancy and characteristic features of the lesion. These features are the vesicular form, the erythema and the pruritus. Some preliminary observations by my colleagues, Drs. Cooke and Coca, seemed to indicate two other striking peculiarities of this condition. Cooke's experiences (unpublished) had resulted in the impression that a relatively high percentage of adults possess a sensitiveness to the active principle of "poison ivy" as shown by experimental contact carried out with a method which will be presently described. A short series of tests carried out by Coca (1) with the same method seemed to indicate that a considerable difference of susceptibility to "poison ivy" existed between adults and children under five years of age. Both of these impressions demanded further investigation before a final decision regarding them could be reached. Such an investigation was undertaken and the results of it are here presented.

There is considerable confusion in the literature as to the nomenclature of "poison ivy." In the present paper we have employed the term *Toxicodendron radicans* (L.), to designate "poison ivy," "climbing ivy" or "three-leaved ivy," thus following the classification of Kuntze as adopted by the United States Herbarium at Washington, D. C., the New York Botanical Garden, and other institutions. This classification appears

to be the most logical, since the word "Rhus," the term used hitherto for the genus, is applied to the members of the Sumac family, that are not poisonous, being replaced by the genus term "Toxicodendron" (Greek, "poison tree"). Under this system of terminology, *Rhus toxicodendron* L. (*Rhus quercifolia* Steud.) "poison oak," eastern species, becomes *Toxicodendron toxicodendron* (L.) Britton. *Rhus vernix* (L.), *Rhus venenata* D. C., "poison sumac," "swamp sumac" or "poison-wood," a plant that is not a true sumac, becomes *Toxicodendron vernix* (L.) Kuntze. We are indebted to Mr. Percy Wilson of the New York Botanical Garden for his assistance in the problem of proper terminology.

Many explanations have been offered as to the specific cause of Toxicodendron poisoning.[1] Burrill (2) believed that poisoning of *Rhus toxicodendron* [*Toxicodendron radicans* (L.), Kuntze,] was due to bacterial growths, but later (3) admitted that his hypothesis had not been proven. Khittel, as quoted by Warren (4), claimed to have found a volatile alkaloid as the cause, while Maisch (5) isolated a volatile acid which he called "toxicodendric acid," and to which he ascribed properties similar to formic and acetic acids. Pfaff (6) proved that toxicodendric acid was in reality acetic acid, and stated that the toxic agent was a non-volatile oily substance which he named "toxicodendrol." Acree and Syme (7) considered the poisonous principle to be a glucoside, yielding rhamnose, fisetin and gallic acid upon boiling with dilute acids. Warren (4), working upon the poisonous principle of *Rhus vernix* [*Toxicodendron vernix* (L.), Kuntze], and McNair (8), studying the poisonous principle of *Rhus diversiloba,* [*Toxicodendron diversilobum* (T. and G.), Greene] were unable to obtain results comparable to those of Acree and Syme. Warren and McNair isolated an acid resin. Stevens and Warren (9) consider the active principle of *Rhus vernix* [*Toxicodendron vernix* (L.), Kuntze] to be a resin which is

[1] The term poisoning is used in this paper as a convenient designation of *Dermatitis venenata* due to "poison ivy," although this condition is obviously not a true poisoning, since the active principle by injection has no effect upon lower animals.

a "clear, amber, oily, red, non-volatile liquid" and "a powerful escharotic."

With the use of a 95 per cent ethyl alcoholic extract of the fresh leaves of *Toxicodendron radicans*, Cooke (10) was able, in 1916, to reproduce the typical vesicular lesion of "poison ivy" in susceptible individuals. Cooke employed the following method of applying the extract for test purposes.

The extract was made by mixing 95 per cent ethyl alcohol with fresh leaves of *Toxicodendron radicans*, which first may be run through a meat chopper. This mixture was allowed to stand for a few days, and the clear extract was obtained by paper filtration. A deposit of some resinous material upon the wall of the flask indicated that the extract represented a saturated solution of the active principle. In our experience, such an extract shows no tendency to a lessening of its activity within at least six or eight months. The extract employed in this investigation was at no time more than four months old.

In the center of the gummed surface of a square of adhesive tape, 5 by 5 cm., there was placed a bit of white blotting paper 0.5 by 0.5 cm., which was then saturated with the alcoholic extract of *Toxicodendron radicans*. The gummed surface of the square of adhesive was then applied to the skin, thus bringing the bit of blotting paper, while still saturated with the alcoholic extract, into intimate contact with the epidermis. We have termed this the "patch test." The flexor surface of the forearm was chosen because of the more delicate texture of the skin of this region, and because of its accessibility. There was no previous preparation of the surface to be tested. After a period of three days the patch was removed, and the area was washed, first with ether to take away any remaining portions of the adhesive, and then with alcohol to remove all traces of the active principle. The skin was inspected for the vesicular lesion typical of ivy poisoning. Every forearm that had been tested was examined twice a week, and differences in the findings were noted. A reaction was considered positive when a typical vesicular lesion of poison ivy was reproduced beneath the patch. No case was considered positive unless there was vesiculation with

erythema and local itching. Plus signs, with the usual interpretation, are employed in the tables to describe the degree of reaction.

The solid residue of a chloroform extract was also employed. This material was applied directly to the skin over an area about 0.5 cm. in diameter. This area was covered with a square of adhesive tape to the center of the gummed surface of which was placed a square of glazed paper about 1 by 1 cm. to prevent absorption of the residue by the adhesive material.

TABLE 1

Comparison of the intracutaneous and patch methods

NUMBER	SEX	INITIALS	DAY OF OBSERVATION									
			Third		Seventh		Tenth		Fourteenth		Seventeenth	
			Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch	Intracutaneous	Patch
66852	F	S. B.	0	+	0	++	0	++	0	++	0	++
67001	F	H. L.	0	+	0	+	0	+	0	+	0	+
72295	F	I. G.	0	++	0	++						
72294	M	A. K.	0	++++	0	++++	0	++++	0	++++	0	++++
69407	F	C. S.	0	+++	0	+++	0	+++	0	+++	0	+++
71003	F	T. D.	0	0	0	0	0	0	0	+	0	+
73603	F	G. O.	0	+++	0	+++	0	+++	0			

At the beginning of this study a comparison was made between the intracutaneous and the patch methods of testing. The usual intracutaneous method was employed in performing these tests. A fine hyperdermic needle was introduced into the layers of the skin, and a small wheal was produced by the injection of about 0.05 cc. of the alcoholic extract of *Toxicodendron radicans*. A control of 95 per cent ethyl alcohol was injected in each case.

It was found in a selected series of seven cases (table 1), that whereas all the cases were positive to the patch test, three being markedly so, in none was there a positive result with the intracutaneous method. In a series of thirty cases, using the ender-

mal (intracutaneous) tests, Strickler (11) asserts that "a positive reaction is indicated by a papule, redness and tenderness at the site of injection," at the end of forty-eight hours, and claims to be able to differentiate between ivy and sumach poisoning with this test. Strickler mentions a list of thirty cases which were successfully diagnosed by this method. His method of preparing the poison ivy extract to be used in the tests, consists of "gathering the fresh leaves of poison ivy and extracting with absolute alcohol, filtering and precipitating. The precipitate is dried and extracted in Soxhlet extractors for ten hours. The extract obtained is dried at low temperature. The toxin is carefully weighed, and dissolved in absolute alcohol, to which a certain amount of sterile water is added to make it non-irritating." This is apparently Syme's (12) method. In our experience the injection of the alcohol alone produced the same effect as did the injection of the extract itself. In both instances, only a papule about 0.5 cm. in diameter with erythema was produced, a lesion quite different from the vesicular lesion of *Dermatitis venenata*. Hence, we considered the intracutaneous test of no value.

DIFFERENCES IN INCUBATION PERIODS

One of the outstanding features of ivy poisoning is the incubation period. In even the most sensitive individuals this feature is present. In the published records of the clinical manifestations of ivy poisoning, there are indications of a difference in the incubation period of this condition in different individuals, but this point has not been systematically investigated. McNair (13) notes that "within twenty-four hours after exposure (to Rhus poisoning) patients frequently break out with a rash. This latent period or period of incubation, is dependent on the slow diffusibility of the poison into and within the skin, as well as in the predisposition of the patient." White (14) has never seen ivy poisoning develop clinically after the fifth day of incubation and in his experience the lesions usually appear within twenty-four to forty-eight hours. Schamberg (15) also thinks that twenty-four to forty-eight hours is the usual clinical incubation period.

As the individuals that were used in the present investigation were kept under continued observation, it was possible to study systematically the differences in the period of incubation. The incubation period was found in some instances to be unexpectedly prolonged. In one individual, in fact, no effect was observed until the twenty-fourth day when a one plus reaction developed at the site of the patch test. This reaction consisted of an area of erythema in which were several elevations not distinctly vesicular. There was definite itching. In the absence of characteristic vesicles there may be some doubt as to the specific nature of this lesion.

The tested individuals have been divided into two groups. Group 1 is composed of those individuals that were observed for a minimum of ten days. The results of the tests of these cases are presented in table 2. Group 2 is composed of those individuals that were observed for only seven days. The results of the tests upon these individuals are shown in table 3. No case was included in either group that was not seen on each consecutive observation day. No cases were recorded that were not observed for at least two consecutive observation days.

In group 1 there are 80 cases, 28 (or 35 per cent) of which were found to be insusceptible to the tests during the entire observation period of 10 to 17 days. These 28 cases were not included in the table (table 2), as they had no incubation period and add no information. If all of the 80 cases had been judged by the results recorded on the third day, 48 cases (or 60 per cent) would have been classed as negative, an error of 25 per cent. If all of the cases had been judged by the third and the seventh day readings only, 40 cases (or 50 per cent) would have been classed as negative, an error of 15 per cent. If these cases had been judged by the third, fifth and seventh day readings, 33 cases (or 41 per cent) would have been judged negative, an error of 6 per cent. From these figures it is seen that the degree of error in the determination of susceptibility by this method drops from 25 per cent on the third day of observation, to 6 per cent on the tenth day of observation. This emphasizes the importance of not judging the reaction from a patch test until after a ten day observation period.

TABLE 2

Group 1. Cases arranged according to length of incubation period

CHART NUMBER	AGE	SEX	INITIALS	DAY OF OBSERVATION						
				Third	Seventh	Tenth	Fourteenth	Seventeenth	Twenty-first	Twenty-fourth
72543	18	M	J. C.	0	0	0	0	0	0	+
76625	48	F	R. K.	0	0	0	+			
70432	21	F	E. K.	0	0	0	+	+	+	
71737	17	F	L. W.	0	0	0	+	+		
70745	42	M	P. V.	0	0	0	+			
70137	44	F	A. B.	0	0	+	+	+		
65139	14	M	A. P.	0	0	+	+	+		
74534	47	M	J. K.	0	0	+	+	0		
66997	10	F	C. B.	0	0	+	+	+		
70728	26	F	E. K.	0	0	+	+	+		
73666	35	M	A. D.	0	0	+	+			
73063	40	M	S. N.	0	0	+	+			
70531	20	M	M. H.	0	0	+	+			
75277	29	M	M. P.	0	+	+	+	+		
71110	45	F	M. G.	0	+	+	+	+		
71090	49	F	M. L.	0	+	+	+	+		
69954	40	F	C. G.	0	+	+	+	+++		
65417	27	M	A. J.	0	+	+	+	+		
70255	31	M	S. S.	0	+	+	+	0		
66107	62	M	L. M.	0	+	0	0			
69844	18	M	E. G.	++++	++++	++++	+			
72294	37	M	A. K.	++++	++++	++++	++++	++++		
65423	8	M	F. B.	+++	++	+	+	+		
61220	24	M	W. M.	+++	+++	++	++	++		
65951	66	M	C. T.	+++	+++	+++	++++	++++		
69407	10	F	C. S.	+++	+++	+++	+++	+++		
71423	20	M	M. M.	+++	+++	+++	++	++		
70256	31	M	P. K.	++	++	++	++	++		
69154	51	F	L. B.	++	+	+	+	+		
75040	45	M	C. L.	++	++	+++	++	++		
66852	44	F	S. B.	++	++	++	++	++		
67001	26	F	H. L.	++	++	+	+	+		
70827	44	F	H. N.	++	++	++	+	+		
62424	40	M	M. M.	++	++	+	+	+		
66616	40	F	I. F.	++	++	+	+	+		
73507	40	F	J. S.	++	+	+	+	0		
62626	12	M	A. O.	++	++	++	+			
73906	30	M	G. B.	++	+	+	+	+		

TABLE 2—*Continued*

CHART NUMBER	AGE	SEX	INITIALS	DAY OF OBSERVATION						
				Third	Seventh	Tenth	Fourteenth	Seventeenth	Twenty-first	Twenty-fourth
63620	10	F	C. R.	+	+	+	+	+		
15660	12	F	L. S.	+	+	0	0	0		
74123	45	F	J. L.	+	+	+	+	+		
66103	22	M	W. S.	+	+	+	++	+		
73514	3	F	M. R.	+	+	+	+	+		
6940	34	M	R. A.	+	+	+	+	+		
67508	28	F	A. M.	+	+	0	0	0		
69572	26	F	E. D.	+	+	+	+	+		
73504	36	F	I. S.	+	0	0	0	0		
71623	28	M	M. S.	+	+	+	+	+		
196	43	F	F. P.	+	+	+				
71615	33	M	J. K.	+	+	+	+++	+++		
65656	60	M	P. C.	+	+	0	0	0		
71827	44	F	H. M.	+	+	+	+			

In group 2 (table 3) there are 24 cases, 13 or 54 per cent of which are negative when classed by the reaction that had developed by the third day only. When judged by both the third and the seventh day observations, 10 or 46 per cent were negative. It will be seen that the results from the third day observations and from the third and seventh day observations in groups 1 and 2 are very similar.

DIFFERENCES IN SUSCEPTIBILITY

It is evident in the results of the tests in all of the tables, that the different individuals are not equally susceptible. In fact, every degree of susceptibility was observed from the least to the greatest. All of the markedly reacting cases had developed the typical lesion by the third observation day. Where the incubation period was longer than three days, the degree of the local test reaction was slight, being one plus in every instance. In three of the most susceptible cases, in addition to the typical lesion produced beneath the patch, lesions upon the arms, hands

and ankles, with general pruritus, had developed within twenty-four hours, that is, before the patch was removed. Close questioning had revealed that these individuals had not been exposed to poison ivy other than by the patch tests. In these individuals, the adhesive plaster was still in place upon the third day, and the area was washed with ether immediately upon its

TABLE 3

Group 2. List of cases observed for 7 days only

CASE NUMBER	AGE	SEX	INITIALS	DAYS OF OBSERVATION	
				Third	Seventh
70528	36	M	M. P.	++++	+++
73766	30	F	A. R.	++	++
75356	34	M	F. R.	+++	+++
75401	28	M	F. P.	++	++
72295	30	F	I. G.	++	++
72879	16	M	A. A	++	++
72554	16	F	E. D.	++	+
73279	24	F	A. T.	++	+
65949	45	M	M. B.	+	+
70816	11	M	V. C.	+	+
62629	47	M	W. R.	+	+
69568	53	M	L. S.	0	0
71086	48	M	E. C.	0	0
70830	45	M	I. W.	0	0
66106	29	F	S. R.	0	0
73579	43	M	W. K.	0	0
68120	48	M	C. B.	0	0
73586	50	M	J. K.	0	0
73416	42	F	A. W.	0	0
74938	7	M	R. F.	0	0
75357	57	M	F. D.	0	+
75399	29	F	D. L.	0	+
75399a	36	F	J. C.	0	+
68809	67	M	J. S.	0	0

removal. The alcoholic extract had been used in the tests and in impregnating the bits of blotter with the extract, an excess was avoided, because such an excess would have prevented the proper adhesion of the patch. These cases, therefore, suggest the possibility that the typical dermatitis of poison ivy develops not only at the point of original contact with the active principle

but also at times upon skin surfaces distant from the primary lesion, by transference of the active substance through the blood and lymph. While this explanation appears plausible, the possibility must be considered that some of the active substance diffused through the adhesive material, in which it is readily soluble, and thus arrived, either at the edges of the patch, or on the external surface of the canvas, from which sites it could be transferred to other parts of the body by external routes.

A correlation of the different degrees of experimental susceptibility noted above, with the degree of clinical susceptibility in the

TABLE 4

Tests upon group of boys

CASE NUMBER	AGE	CLINICAL HISTORY	CLINICAL ONSET	DAY OF OBSERVATION		
				Third	Seventh	Fourteenth
109	14	General, face and body	2	++++	++++	++
112	15	General, face and body	2	+	+	0
117	20	Severe on hand and feet	2	+	+	0
114	16	Severe on hands	2	+++	+++	+
115	17	Severe on feet	2	++++	++++	++
116	18	Slight	2	++	++	0
110	15	Slight	2	++	++	0
111	15	Slight	3	+	+	0
108	14	Slight	2	++	+	0
106	13	Slight, on extremities	3	+	+	0
105	13	Negative	0	+	+	0
107	14	Negative	0	0	0	0
113	15	Negative	0	0	0	0

same individuals, could not be undertaken in these series since most of the individuals were of foreign birth, and were not acquainted with poison ivy or its natural effects. However the opportunity for making such a correlation was offered in a group of boys. The protocol of these comparative observations is presented in table 4. The group consisted of thirteen boys between thirteen and twenty years of age, who, in midsummer, pitched camp at a site covered with poison ivy vine. Ten of the thirteen gave the clinical history of ivy poisoning as the result of this experience. The tests, in two of the three clini-

cally negative cases, were negative. The third clinically negative case gave a faintly positive reaction (one plus) by patch test on the third day, but this reaction was as pronounced as in four other cases clinically positive, in two of whom the attack was mild, in two, severe. All of the cases giving severe test reactions had suffered severe poisoning while in camp. Two other cases that had been severely affected while in camp gave only faintly positive reactions. No individual that had exhibited any degree of clinical susceptibility was negative on test. The clinical incubation period of all the cases was practically the same, the dermatitis appearing upon the evening of the second day in all but two of the cases. In these two cases the lesions occurred upon the morning of the third day.

In general it can be said that, with the kind of extract employed, there is a degree of parallelism between the results of the tests and the clinical history of the cases.

AGE INCIDENCE

Of the eighty individuals included in group 1, 52, or 65 per cent, responded with a positive reaction to the patch test. All but one of these individuals were eight years of age, or over. The number of tested individuals in the different age periods is too small to permit a definite conclusion as to a possible difference in susceptibility in poison ivy among individuals over eight years of age. For purposes of comparison however, we may take 65 per cent as representing approximately the average susceptibility of the individuals over eight years of age with the technic, and with the use of the alcohol or chloroform extracts described above. Through the courtesy of Dr. F. H. Bartlett opportunity was afforded to apply the patch tests in 18 infants between five weeks and eighteen months of age, all patients in Dr. Bartlett's service at the Babies' Hospital, New York City. The results of these tests are shown in table 5. Two tests were carried out in each case. On one arm the alcoholic extract was applied by means of the patch test. On the other arm the residue of the chloroform extract was employed in the manner previously described. It is seen that no positive reaction occurred by either test.

TABLE 5

Tests upon infants

CASE NUMBER	AGE	SEX	DAYS OF OBSERVATION		
			Third	Seventh	Tenth
	months				
118	$1\frac{1}{4}$	F	0	0	Home
119	2	F	0	0	0
120	$4\frac{1}{2}$	M	0	0	0
121	5	M	0	0	Home
122	5	M	0	0	0
123	6	M	0	0	0
124	6	F	0	0	0
125	$6\frac{1}{2}$	M	0	0	Home
126	7	F	0	0	0
127	7	F	0	0	0
128	8	F	0	0	0
129	8	M	0	0	Home
130	$10\frac{1}{2}$	M	0	0	0
131	11	M	0	0	0
132	11	F	0	0	0
133	12	F	0	0	0
134	12	F	0	0	Home
135	16	F	0	0	0
136	18	M	0	0	0

SUMMARY

1. The typical vesicular lesion of *Dermatitis venenata* can be produced by means of an alcoholic or chloroform extract of the fresh leaves of *Toxicodendron radicans*, applied to the skin surface.

2. In our hands, the typical vesicular lesion of *Dermatitis venenata* could not be produced by the intradermal injection of an active alcoholic extract. The lesion thus produced was not different from that caused by the intradermal injection of the solvent.

3. With the patch test, differences can be demonstrated in the susceptibility of different individuals to poison ivy, and in the incubation period of the lesion.

4. With the technic used, infants could not be shown to be susceptible.

REFERENCES

(1) Coca, A. F.: Jour. Immunol., 1922, **7**, 193.
(2) Burrill, J. T.: Am. Assoc. Adv. Sci. Proc., 1882, **31**, 515.
(3) Burrill, J. T.: Garden and Forest, 1895, **8**, 368.
(4) Warren, L. E.: Pharm. Jour., 1909, **83**, 531.
(5) Maisch, J. H.: Am. Pharm. Assoc. Proc., 1865, **13**, 166.
(6) Pfaff, F.: Jour. Exp. Med., 1897, **2**, 181.
(7) Acree, S. F., and Syme, W. A.: Am. Chem. Jour., 1906, **36**, 301.
(8) McNair, J. B.: Jour. Am. Chem. Soc., 1916, **38**, 1417.
(9) Stevens and Warren: Am. Jour. Pharm., 1907, **79**, 518.
(10) Cooke, R. A.: From unpublished clinical studies.
(11) Strickler, A.: Jour. Amer. Med. Assoc., 1921, **77**, 910.
(12) Syme, W. A.: Some constituents of poison ivy. Johns Hopkins Univ. Dissertation, 1906.
(13) McNair, J. B.: Jour. Infect. Dis., 1916, **19**, 419.
(14) White, J. C.: Jour. Cutan. Dis., 1906, **30**, 280.
(15) Schamberg, J. F.: Jour. Cutan. Dis., 1906, **30**, 280.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

VII. THE AGE INCIDENCE OF SERUM DISEASE AND OF DERMATITIS VENENATA AS COMPARED WITH THAT OF THE NATURAL ALLERGIES

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Any classification of the various forms of hypersensitiveness must be considered tentative so long as the mechanism of these phenomenona remains unknown. In the absence of this knowledge, however, it is useful to group the phenomena according to the facts at hand. Thus, anaphylactic hypersensitiveness can be properly separated from all the other forms because there is no satisfying evidence that these latter are dependent upon an antibody-antigen mechanism, as is the former.

In a previous publication the author associated the drug, food and animal idiosyncrasies, hayfever, asthma, serum disease and dermatitis venenata under the term allergy. Two of these conditions are distinguished from the others by noteworthy peculiarities. Serum disease presents the almost constant characteristic of the incubation period and dermatitis venenata differs from the others in which the skin is involved, in the constant and characteristic nature of the lesion. It has been recently found, also, that serum disease differs strikingly from most of the other forms of human hypersensitiveness in its percentage incidence. The observations of Longcope and Mackenzie and of Rufus Cole supported by statistics collected in the Boston City Hospital (1) indicate that about 90 per cent of the white race are susceptible to ordinary serum disease upon *intravenous* administration of large quantities of horse serum. A high percentage of susceptibility also to poison ivy is indicated by some

experiments by Cooke and by the writer; which will be presently described. According to Cooke and Vander Veer (2), on the other hand, only about 10 per cent of the white race are subject to the natural allergies.[1]

These differences are all so wide that they suggest a difference in the underlying mechanism of the respective phenomena, notwithstanding the apparent association of all of them as revealed in the general lack of susceptibility in the American Indian (1).

It is the purpose of the present communication to describe an additional difference between the natural allergies and the two other forms of human hypersensitiveness as well as a certain apparent difference between these latter two.

In their notable paper on the "human sensitizations," Cooke and Vander Veer (2) showed that the age incidence of hayfever, asthma and the food, drug and animal idiosyncracies is governed by heredity. For example, if both parents are affected, 36.3 per cent of the potentially susceptible offspring begin to exhibit symptoms within the first five years of life. This figure is in significant contrast with the percentage incidence for this period of life among potentially susceptible individuals whose antecedent family history is *negative* with respect to natural allergies; that incidence is only 5.1 per cent.

From table 2 of the paper by Cooke and Vander Veer can be calculated the age incidence of all potentially susceptible individuals without regard to the family history This incidenced taken in successive five year periods is 11.6, 16.2, 15.2, 11.6, 13.8. 11, 9.8, 6, 1.2 per cent, with 3.6 per cent for the entire period over 45 years. With these figures we can see how many of one generation of potential susceptibles have begun to exhibit symptoms in the different age periods by taking the sum of the

[1] In this paper the expression "natural allergies" will be used for the sake of convenience to designate those forms of human hypersensitiveness which result from natural contact with the exciting agent. For the writer's convenience, also, although quite illogically, the susceptibility to poison ivy will be excluded from this group, which will comprise those characterized by the symptoms of hayfever, asthma, multiform eruption, angio-neurotic edema and some other symptoms after natural contact.

individual percentage for all of the preceding age periods. Thus, 11.6 per cent of the potential susceptibles present symptoms by by the fifth year; 27.8 per cent present symptoms by the tenth year; 43 per cent are affected by the fifteenth year and so on up to the last age period of over 45 years when all of the potential susceptibles or 100 per cent have become affected.

Thus, it is evident that as a generation advances in years the number of its members affected with the natural allergies constantly increases.

The percentages taken from table 2 of Cooke and Vander Veer are calculated with reference to the number of potentially susceptible individuals and not to the whole number of individuals in the generation. However, it is evident that if the corresponding percentages could be calculated with reference to the entire generation these would be merely much smaller than those referring to the susceptible group; their mathematical ratios to one another would remain unchanged.

In other words, the percentage of individuals affected with the symptoms of the natural allergies increases considerably in each generation, at least in the early successive life periods. Thus the percentages in the second and third life periods (27.8 and 43) are respectively about 2½ and 4 times as great as that of the first life period (11.6).

With these facts as a basis of comparison, a study of the age incidence of serum disease and of dermatitis venenata (poison ivy) was undertaken.

The data used in the study of serum disease were obtained in the Durand Hospital of the John McCormick Institute for Infectious Diseases in Chicago (service of Dr. G. H. Weaver) and in the Municipal Hospital in Philadelphia. We are indebted to Dr. L. Hektoen for permission to use the Durand Hospital records and to Miss Adeline S. Lorge for collecting the data in that hospital. Acknowledgment is due, also, to Mr. Joseph Peacock and to Miss Helen I. Barrett, who collected the Philadelphia data.

In table 1 the cases in the two institutions are arranged in five divisions according to age. In the first column of each division

is given the total number of individuals receiving injections of serum; in the second column is the number of those in whom serum disease was noted; in the third column is the percentage incidence of serum disease.

Differences in the percentage incidence in the different life periods are indicated in the two series of cases. However, it seems significant that those differences are much greater in the Chicago series than they are in the relatively much larger Philadelphia series and that when the two series are combined the

TABLE 1

Showing the age incidence of serum disease

	0–5 YEARS			6–10 YEARS			11–15 YEARS			16–25 YEARS			25 YEARS UP		
	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected.	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease	Number of cases injected	Number of cases showing serum disease	Percentage incidence of serum disease
Chicago...	425	75	17.9	314	65	20.7	136	17	12.5	190	42	22.1	142	21	14
Philadelphia....	2215	195	8.8	1498	161	10.8	331	37	11.1	376	36	9.6	233	18	7.7
Total ..	2640	270	10.2	1812	226	12.5	467	54	11.6	566	78	13.8	375	39	10.4

differences are reduced to relative insignificance. It seems probable, in other words, that as the number of individuals under observation in the different age groups increases, the differences in the percentage incidence will tend to diminish.

It must be freely admitted that the clinical records of the incidence of serum disease are practically never perfect. However, it seems most unlikely that this inaccuracy could be much greater for one age period than for another. Hence, the conclusion may be confidently drawn from the foregoing statistical study of serum disease that the incidence of this condition is not materially different in the different age periods.

The determination of the age incidence of sensitiveness to poison ivy is attended with the difficulty that many individuals either have not come in contact with this plant or have not recognized the nature of the lesion which resulted from such contact. Trustworthy data in these circumstances could be obtained only by experiment. This was carried out by applying an extract of the leaves of poison ivy to a small area of the skin of a number of individuals and observing the effect after 48 hours contact. The extract was made by grinding the young leaves in a meat-chopper and mixing the resulting moist mass either with 95 per cent alcohol or with chloroform.

Both of the extracts were filtered through paper after three days contact with the ground leaves. The alcoholic extract was used in the crude fluid form. The chloroform extract was placed in a broad shallow dish before an electric fan until all of the chloroform had been evaporated and only a sticky residue remained.

These extracts were applied to the skin according to a method employed by Robert A. Cooke in some unpublished experiments and referred to by him as the "patch" method or test. The fluid alcoholic extract was applied to the skin by soaking a small square of blotting paper with it and keeping the impregnated paper in contact with the skin with the use of adhesive-plaster.

The residue of the chloroform extract was smeared on a small area of the skin over the anterior surface of the forearm and the area was protected with the use of adhesive plaster. Care was taken to prevent contact of the adhesive preparation on the plaster with the ivy extract as the latter is very soluble in the adhesive material and is quickly taken up into it from the skin surface.

Observation of the individuals so treated was continued until a lesion appeared or at least for five days. The test was carried out upon twelve adults over 20 years of age and of these only 1 remained unaffected. The lesions that developed in the eleven susceptible individuals resembled those of dermatitis venenata in their vesicular nature and in the itching; they remained local.

The high percentage of susceptibility among these adults is in agreement with that noted by Cooke in the unpublished experiments referred to above.

As soon as these results were obtained the test was applied in a series of twelve children five years of age or younger. Two of these were under one year; the others ranged between three and five years. Only one of these was found susceptible to the ivy extract—a boy of five years. These children were patients in the New York Hospital in the service of Dr. J. C. Roper, who kindly permitted the tests to be carried out and who controlled the results.

At this point the investigation was interrupted but it was thought that the results of even these two short preliminary series give sufficient indication of a great difference in susceptibility to poison ivy between young children and adults. This indication is rendered a practical certainty by the findings of Dr. Spain (3).

From these experiments it seems that the age incidence of poison ivy susceptibility differs from that of both the "natural" allergies and serum disease.

The susceptibility to serum disease is exhibited by a constant proportion of individuals in all of the life periods. That to the "natural" allergies is shown by a proportion of individuals which increases rapidly in the early five-year periods. On the other hand, while the susceptibility to poison ivy is exhibited by a proportion of individuals which increases from birth to adult life, like that to the "natural" allergies, it differs strikingly from the latter in the very high percentage susceptibility of the adults—about 90 per cent as contrasted with the 10 per cent general incidence found by Cooke and Vander Veer (2). This difference is really increased by the fact that the 10 per cent incidence of the natural allergies found by these authors includes susceptibility to a number of substances; it cannot, therefore, be fairly compared with the incidence of susceptibility to the single substance—poison ivy.

It is not possible to interpret the differences that have been found among the different forms of human hypersensitiveness

referred to. While those differences appear to indicate a different origin or mechanism of the three forms, judgment in this question must await further knowledge.

SUMMARY

1. Serum disease differs from other forms of human hypersensitiveness in the almost constant characteristic of the incubation period and in its high percentage incidence.

2. Dermatitis venenata differs from the other forms of human hypersensitiveness in which the skin is affected in the constant and characteristic nature of the lesion.

3. Statistical study and some experiments reveal the following differences in the age incidence of the "natural" allergies, serum disease and dermatitis venenata:

a. The age incidence of the "natural" allergies increases rapidly in the early age periods but probably does not greatly exceed 10 per cent in any period.

b. The age incidence of dermatitis venenata increases greatly from childhood to adult life and reaches a high percentage (probably about 90 per cent).

c. The age incidence of serum disease seems not to change during life.

REFERENCES

(1) Coca, A. F.: Jour. Immunol., 1922, **7**, 163.
(2) Cooke, R. A., and Vander Veer, A., Jr.: Jour. Immunol., 1916, **1**, 201.
(3) Spain, W. C.: Jour. Immunol., 1922, **7**, 179.

STUDIES IN SPECIFIC HYPERSENSITIVENESS

VIII. ON THE RELATIVE SUSCEPTIBILITY OF THE AMERICAN INDIAN RACE AND THE WHITE RACE TO THE ALLERGIES[1] AND TO SERUM DISEASE

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The fact that serum disease generally appears after an incubation period following the injection of an antigenic protein and is usually accompanied by the formation of specific antibody (precipitin) would seem to separate this form of human hypersensitiveness from those which are exhibited immediately upon natural contact with the exciting agent in the entire absence of demonstrable antibodies. The former condition seems to be experimentally induced and dependent merely on a suitable previous contact with the exciting agent—serum protein: the latter, according to the studies of Cooke and Vander Veer, appear to be wholly subject to an hereditary factor and not dependent upon previous contact with the exciting agent.

Our knowledge of these conditions is drawn from observations upon the white race. Certain publications, to which we shall refer again, seemed to indicate that some of the forms of hypersensitiveness in human beings (asthma, dermatitis venanata) are rare among the American Indians and it was thought that investigation of this apparent racial difference might produce some information regarding the nature of the different forms of specific human

[1] By allergy is meant the inherited forms of specific human hypersensitiveness, such as hay-fever, asthma, angio-neurotic edema and urticaria.

hypersensitiveness as well as some indication of the relation of the American Indians to the other human races.

Particularly, it seemed desirable to find out first, whether the American Indian is relatively infrequently affected by the inherited forms of hypersensitiveness such as asthma and hay-fever and secondly, whether or not in such case the induced condition of serum disease is also infrequent in that race.

If the incidence of serum disease were found to be equal in the Indian race and in the white race, this form of human hypersensitiveness would be, thereby, the more sharply differentiated from the inherited form. If, on the other hand, both forms of hypersensitiveness were found to be equally infrequent, then an identity in at least a part of the underlying mechanism of the two forms would be indicated.

Through correspondence with physicians that had had extensive medical experience among American Indians we learned that hay-fever, asthma, urticaria and drug allergy are very uncommon in the full blooded individuals of that race. We are able, with the coöperation of Dr. N. P. Sherwood, Professor of Bacteriology in the University of Kansas, and Mr. H. B. Pearis, Superintendent of Haskell Institute in Lawrence, Kansas, to observe the occurrence of serum disease in twenty-six students in Haskell Institute, all listed as full blooded American Indians.

THE OCCURRENCE OF THE ALLERGIES AMONG AMERICAN INDIANS

A search of the literature has revealed but few references to the incidence of allergic conditions in the Indians.[2] In his report on Physiological and Medical Observations among the Indians of Southwestern United States and Northern Mexico (1), Aleš Hrdlička remarks on page 188, "No instances of severe asthma were encountered," and on page 191, asthma is "rare among Southwestern Indians."

In his article on "Skin Diseases among Full Blooded Indians of Oklahoma" (2), E. S. Lain writes:

[2] In this article the word Indian always refers to the American Indian.

The extreme rarity of dermatitis medicamentosa, dermatitis venenata, may partially be accounted for by the lack of common usage of drugs, soaps, and other chemicals, though the Indians frequently apply quite freely on their faces and other exposed parts paints and dyes made from the wild plants, berries, etc. The skin of the Indian is apparently almost immune to the poison ivy and other plants which cause so much discomfort to the women of our race.

Our own inquiry was made in the form of a questionnaire, answers to which were received from eleven physicians and one superintendent of an Indian school. The replies were based on extended medical observation of about 40,000 full blooded Indians.

Those replying to the questionnaire were:

Dr. A. J. Anderson, Lawrence, Kansas.

Mr. Clyde M. Blair, Superintendent Chilocco Indian School, Chilocco, Oklahoma.

Dr. A. D. Lake, Gowanda, New York.

Dr. Otis E. Lovelady, Ponca Agency, Whiteagle, Oklahoma.

Dr. A. E. Marden, W. S. Indian Vocational School, Phoenix, Arizona.

Dr. James M. Meason, Pima School, Sacaton, Arizona.

Dr. Charles M. Ming, Okmulgee, Oklahoma.

Dr. W. W. Rublee, Sherman Institute, Riverside, California.

Dr. F. A. Spafford, Flandreau, South Dakota.

Dr. A. J. Wheeler, W. S. Indian Vocational School, Phoenix, Arizona.

Dr. Lawrence White, Keshena, Wisconsin.

Dr. A. M. Wigglesworth, Albuquerque, New Mexico.

The essential questions proposed were:

1. Have you observed the occurrence of asthma or hay-fever in full-blood Indians?

2. Do these conditions occur in the white population of the vicinities in which the observations upon the Indians were made?

3. Have you observed the occurrence of urticaria in full-blood Indians?

In all of the replies question 2 was answered in the affirmative.

The following men could recall no instance of asthma, hay-fever or urticaria in a full-blood Indian: Mr. Blair, Drs. Lovelady, Meason, Anderson, E. F. Menger, Ming, Roblee, Spafford, Wheeler, and White.

Dr. Marden has seen "two or three cases of asthma," and "numerous cases of urticaria" in full-blood Indians (about 10,000 individuals under observation).

Dr. Menger has seen "hay-fever, asthma, urticaria and dermatitis venenata *only* in the breeds" (about 1500 individuals under observation).

Dr. Wigglesworth has seen hay-fever and asthma in full-blood Indians and "urticaria many times." (About 15,000 individuals under observation.)

Dr. Lake has seen "a few cases of hay-fever and of asthma." He states that "hives are common and poisoning from ivy is frequent." However, he writes that "very few" of the Indians under his observation are full-blooded.

These replies suggest that while the allergic trait is not absent in the Indian race this character is very much less marked in the Indian than it is in the white race.[3] The observation of allergic symptoms in some pure Indians makes it inadmissible to assume the complete absence of the basic condition in all of the Indians that do not naturally present the outward signs of it.

It may be that, exactly as in the white races, some individuals are hypersensitive in a degree too slight to allow the development of symptoms upon the *natural contact* with the exciting agent. In such individuals the *injection* of the agent would reveal the latent condition.

From the foregoing facts it could have been anticipated that if serum disease is associated in some way with the allergies it should occur in full-blood Indians upon injection of serum, but much less frequently and possibly in milder form than it does in the white race. In fact, this was the outcome of the experiences which we are about to describe.

[3] In the same inquiry the statement of Lain regarding the relative insusceptibility of the Indians to "poison ivy" was confirmed.

We had learned from Dr. W. T. Longcope that in his experience symptoms of serum disease occur in at least 90 per cent of white individuals receiving 100 cc. or more of whole horse serum by intravenous injection.

With this high percentage as a standard of comparison it was thought that at least some indication of the relative susceptibility of the two races to serum disease could be obtained from the results of the intravenous injection of about 100 cc. of normal horse serum into even a few of the Indians.

The observations were made upon twenty-six full-blooded Indians, all students in Haskell Institute situated in Lawrence, Kansas. We consider it a privilege to record the fearlessness and patience with which these intelligent young men, most of whom were fully aware of the investigatory nature of the procedure, submitted to the injections. It is not possible to distinguish any individuals among them in the respect of courage or fortitude. Those who presented themselves in the first series faced uncertainty as to the possible pain and danger attending the injections. Those that came later came with the knowledge of the unpleasant symptoms which some of their predecessors had suffered. It should be recorded that the two who were the last to be injected had witnessed the effect of the injection upon Charlie Hutchison. These two were given ample opportunity to withdraw, but neither would take advantage of that suggestion.

THE OCCURRENCE OF SERUM DISEASE IN THE AMERICAN INDIAN

The material injected was fresh normal horse serum preserved with the addition of 0.6 per cent of a mixture of equal parts of "Three Cresols" and ether. According to Krumwiede and Banzhaf (3), one of the unpleasant immediate effects (chills) of the injection of serum preserved with cresol is avoided by adding the preservative mixed with an equal volume of ether. It may be stated here that this symptom was absent in all but one of the individuals in the present series (Jesse King).

The serum was prepared especially for this investigation in the laboratories of the H. K. Mulford Company and delivered

to us in double stoppered 110 cc. tubes together with a number of the H. K. Mulford Company intravenous outfits.[4] With these convenient outfits we were able to make the injections at the rate of six in an hour.[5]

Previous to the injections cutaneous tests were made with dried horse serum prepared by the H. K. Mulford Company. All of these resulted negatively.

The serum was brought to body temperature by placing the tubes in warm water.

The effects of the injections in the different individuals were as follows:

1. William Frank (Creek). Received 110 cc. Slight headache on the day of injection and temperature of 99.8F. Presented no other symptoms at any time.[6]

2. Joe Bearhead (Cheyenne). Received 110 cc. Complained of headache on day of injection. Had some epistaxis on the third day. On the 10th day there were a few urticarial spots on the arms and body without elevation of temperature; these were present also on the 11th day still without fever, but they had disappeared by the 12th day.

3. Benjamin Osage (Cheyenne). Received 110 cc. Immediately after the injection a swelling 1 cm. in diameter appeared on the left forearm just above the wrist. The swelling was situated more deeply than the usual urticarial wheal. A smaller spot appeared on the forehead and another 2 mm. in diameter above the one on the forearm. There was severe headache on the day of the injection with a temperature of 100°F. There were no further symptoms.

4. Harber Johnson (Creek). Received 110 cc. There was immediate injection of the conjunctiva with some swelling of the eyelids and face. There was headache on the day of the injections, but no symptoms thereafter.

5. William Atkins (Otoe). Received 25 cc. There were no symptoms at any time.

[4] We are under great obligation to Drs. John Reichel, F. M. Huntoon and J. A. Murphy of the H. K. Mulford Company for the care with which the serum was prepared and delivered to us.

[5] Acknowledgement must be made of the skillful assistance of the nurses—all Indian girls—under the direction of Miss Anderson, superintendent of nurses in the Institute.

[6] There was some coughing during the injection or immediately afterward in most instances and in some a slight sense of oppression in the chest.

6. Thomas Wasson (Baiut). Received 110 cc. Within ten minutes after the injection two urticarial spots appeared on the eyelid and the neck. There were no further symptoms.

7. Delmar Scott (Mojave). Received 110 cc. There were no symptoms until the 8th day when there was fainting and a temperature of 100°F. On the 9th day generalized urticaria appeared which lasted three days. There was no elevation of temperature on the 9th day nor thereafter.

8. Robert Leve Leve (Walapi). Received 110 cc. There were no symptoms until the 12th day when there were severe joint and muscle pains without temperature elevation or eruption.

9. William Hampton (Choctaw). Received 110 cc. Complained of joint pains on the 5th day and again on the 10th day when some urticarial spots appeared. The joint pains continued on the 11th and 12th days but there was no urticaria after the 10th day. There was no elevation of temperature at any time.

10. Lawton Raymond (Navajo). Received 110 cc. There were no symptoms at any time.

11. Joseph Parnell (Assiniboin). Received 110 cc. There were no symptoms at any time.

12. Wallace Littlefinger (Sioux). Received 110 cc. On the 11th and 12th days there was urticaria without fever and on the 13th day there was a temperature of 99°F. with joint pains but without urticaria.

13. Edward Davenport (Sac and Fox). Received 110 cc. There were no symptoms at any time.

14. Emery Redbird (Ottawa) Received 110 cc. There were no symptoms at any time.

15. Morris Baken (Choctaw). Received 20 cc. On the 11th day there was swelling of the left parotid gland without fever. On the 12th day there were joint pains with a temperature of 100°F. No symptoms thereafter.

16. William Ruskin (Navajo). On account of the very small size of the vein a double puncture occurred causing a hematoma, which prevented the injection.

17. Marion Runsthru (Assiniboin). Received 110 cc. There were no symptoms at any time.

18. Andy Snap (Creek). Received 110 cc. On the 6th day there was urticaria without fever; on the 7th day the urticaria had disappeared but there were cramps and weakness with a temperature of 99°F. Urticaria reappeared on the 8th day accompanied by edema of the face and

a temperature of 100°F. There were no symptoms on the 9th day nor thereafter.

19. Jesse King (Creek). Received 95 cc. There was immediate marked uneasiness with pain in the chest and about the eyes. Pulse at eight minutes 140; at thirteen minutes 118, and at eighteen minutes 104. At thirty minutes he was able to walk out. At one hour there was a chill. There were no further symptoms.

20. Mason Kawaykla (Apache). Received 110 cc. There was urticaria on the 10th and 11th days without fever or other symptom.

21. Walter Emarthla (Creek) Received 110 cc. There were no symptoms at any time.

22. James Foster (Creek). Received 110 cc. There were a few urticarial spots on the 10th day without fever or other symptoms.

23. Abel Archibald (Creek). Received 110 cc. There were no symptoms at any time.

24. John Alonzo (Pueblo). Received 90 cc. Vomited immediately after the injection, but presented no further symptoms at any time.

25. Charles Hutchinson (Arapahoe). Received 80 cc. Immediately complained of pain in the chest. There was edema of the eyelids and congestion of the conjunctivae; a general weakness and a rapid and weak pulse (116) five minutes after the injection. Had to be carried to bed There was severe vomiting after two hours. The edema of the eyelids and the weakened heart action persisted into the following day. Thereafter there were no further symptoms. There was a previous history, in this case, of a weak heart and a constitutional weakness.

26. Andrew Juan (Pima). Received 110 cc. There was headache and a temperature of 100°F. on the 2nd day, but no further symptoms at any time thereafter.

27. Edward Meeks (Arapahoe). Received 110 cc. There was a slight malaise immediately after the injection and on the 9th day urticaria without elevation of temperature.

In considering what symptoms were to be regarded as indicative of "serum disease" we have been guided, naturally, by the attitude of those upon whom we depended for our "control" series among the white race, that is Dr. W. T. Longcope and Dr. Rufus Cole. These observers have used only eruption and joint pains with or without fever as criteria of that condition, and as is customary they have ignored the immediate effects of the injections, excepting eruption. We have, therefore, omitted

from consideration the effects noted in the cases of William Frank, Harber Johnson, Jesse King, John Alonzo, Charles Hutchison and Andrew Juan.

In table 1 is given a summary of the symptoms that could be regarded as those of "serum disease" resulting from the injections into the Indians.

It is seen that the incidence of the condition among those injected was 46 per cent; the average duration of the symptoms was two days and the average elevation of temperature was 0.38°F.

In comparing the course of serum disease as just described in the Indians with that in the white race it must be pointed out that the injections made in the Indians were given to healthy young men who were under orders to report to the physician in charge (Dr. E. F. Menger) the slightest ailment. For this reason the existence of serum disease was recognized in several of the Indians by symptoms (such as joint pains and edema and by the immediate appearance of a few urticarial spots) which are rarely noted in the usual medical history. It was necessary, therefore, in order to obtain comparable statistics among white individuals to seek them in institutions in which the occurrence of serum disease is given particular attention. These requirements were amply met in the records of a series of cases in the Presbyterian Hospital of New York City which were under the care of Dr. W. T. Longcope and Dr. George M. Mackenzie. We are indebted to these observers for permission to publish the data and to Dr. E. P. Maynard by whom the data were collected.

It is evident that the relative susceptibility of the two races should not be judged solely by the relative percentage incidence. A fair comparison should include also the factors of duration of the disease and its severity. As an index of severity we have used the single feature of elevation of temperature. In a series of fifty-two individuals treated with serum (horse) injections in the Presbyterian Hospital and observed continuously for one month, serum disease occurred in forty-eight instances—an incidence of 92.4 per cent.

TABLE 1

Showing the clinical course of serum disease in full blooded Indians

	DAY OF INJECTION									
	1st	5th	6th	7th	8th	9th	10th	11th	12th	13th
Baken (Choctaw)								Swelling of parotid. 98.6°F.	Joint pains. 100°F.	0
Littlefinger (Sioux)								Hives. 98.6°F.	Hives. 98.6°F.	No hives. Joint pains. 99°F.
Kawaykla (Apache)							Hives. 98.6°F.	Hives. 98.6°F.	0	
Foster (Creek)							Hives (few). 98.6°F.	0		
Hampton (Choctaw)		Joint pains. 98.6°F.					Joint pains. Few hives. 98.6°F.	Joint pains. No hives. 98.6°F.	Joint pains. No hives. 98.6°F.	0
Snap (Creek)			Hives. 98.6°F.	Cramps. Weakness. No hives 99°F.	Hives. Edema face. 100°F.	0				

Meeks (Arapahoe).......						Hives. 98.6°F.	0			
Scott (Mojave)..........					Weak-ness. 100°F.	General hives. 98.6°F.	Hives. 98.6°F.	Hives. 98.6°F.	0	
Leve Leve (Walapi).....									Joint pains. No hives. 98.6°F.	0
Bearhead (Cheyenne)...							Few hives. 98.6°F.	Few hives. 98.6°F.	0	
Osage (Cheyenne).......	Immediately, few urticarial spots. No further symptoms.									
Wasson (Baiut).........	Immediately, few urticarial spots. No further symptoms.									

In table 2 the duration of the condition in forty-two of the cases is given and for direct comparison the duration of the condition in the twelve Indians. The average duration in the white individuals was four and one-half times as great as it was in the Indians.

As an index of the relative severity of the serum reaction, comparison has been made of the average elevation of temperature above the normal level.

TABLE 2

Duration of serum disease after intravenous injection of serum

IN 42 WHITES (PRESBYTERIAN HOSPITAL) NEW YORK CITY						IN 12 FULL-BLOODED INDIANS (HASKELL INSTITUTE)	
Days	Cases	Days	Cases	Days	Cases	Days	Cases
1	1	9	1	15	2	1	5
3	3	10	1	16	1	2	3
4	11	11	1	20	2	3	1
5	6	12	1	23	1	4	3
6	2	13	1	24	2		
7	4	14	1	25	1		
Average duration 9 days						Average duration 2 days	

In determining the average elevation of temperature in the white individuals only those cases were considered in which the temperature had been normal before the symptoms of serum disease had appeared. As all of these individuals had been suffering from a bacterial infection they were, according to Dr. Mackenzie, who selected the cases, possibly more prone to temperature elevation than a normal individual. Dr. Mackenzie suggests, therefore, that this instability be accounted for in estimating the temperature elevation in these individuals by advancing the normal level somewhat. As the temperatures in these cases, were taken per rectum, Dr. Mackenzie suggests that 100°F. be taken as the normal temperature. This suggestion has been adopted.

Following are the highest rectal temperatures observed in thirty-seven of the Presbyterian Hospital cases during the period in which the individuals presented symptoms of serum disease:

104.0	101.0	104.8
100.6	99.8	101.6
103.0	99.4	100.0
100.6	103.6	101.2
101.0	103.0	102.2
103.0	99.6	100.0
100.0	100.0	104.0
100.2	102.4	99.6
100.2	100.0	100.0
99.8	100.2	99.2
100.2	99.4	102.0
101.2	100.4	
99.8	102.2	

The total elevation of temperature over 100°F. in all of these individuals was thus 42.6°, or an average elevation of 1.15°F. The total elevation of temperature over 98.6°F. (by mouth) in the twelve affected Indians was 4.6°F., or an average elevation of 0.38°F.

TABLE 3

Comparison of the serum reactions of the white and Indian races

	WHITE	INDIAN
Serum-treated cases followed completely	52	26
Cases of serum disease	48	12
Percentage incidence	92.4	46
Average duration (days)	9−	2
Average temperature elevation, degrees F	1.15	0.38

Ratio in which the two races are affected:

$$\frac{92.4}{46} \times \frac{9}{2} \times \frac{1.15}{0.38} : 1 = 27:1$$

In table 3 is presented a summarized comparison of the occurrence of serum disease in the two races. The ratio in which the two races are affected is properly estimated by multiplying together the single ratios of the three different factors—incidence, duration and severity. By this calculation the susceptibility of the white race is found to be about twenty-seven times as great as that of the Indian.

Objection must be made to the placing of much dependence upon the figures used in this calculation on account of the small-

ness of the number of individuals in both of the series that form the basis of comparison. In the circumstances, access cannot be had to further data as to serum disease in the Indian. However, we are able by the courtesy of Dr. Rufus Cole to supplement the observations in the white individuals with his own experiences in the Hospital of the Rockefeller Institute. It was important to obtain further data upon the white race because of the surprisingly high percentage of incidence observed in the Presbyterian Hospital—the highest, by far, that has yet been reported. We quote here from a personal communication from Dr. Cole:

No intensive study of serum disease has been made at The Hospital of The Rockefeller Institute and any statistical conclusions drawn from our experience cannot be considered final. However, I have collected 223 cases in which amounts of serum varying from 30 cc. to 2000 cc. were administered. Only a few of the patients, however, received the very large amounts; most patients received from 200 cc. to 400 cc. during a period of two to three days. We have records concerning the occurrence of urticaria, skin rash, enlarged glands, red and painful joints, and fever. The statistics regarding fever will have to be studied much more carefully before much stress can be laid upon them, since in many pneumonia patients who have received serum, it is impossible to say whether a late elevation of temperature is the result of the administration of the serum or is due to some complication.

Among the 223 cases, the occurrence of a skin rash, usually urticaria, at some time following the injection of serum, was noted in 158 patients; i.e. in 70 per cent. The rash appeared from one to thirty days following the injection of serum and varied in degree from a few urticarial wheals to urticaria covering almost the entire body. In a number of cases there occurred an erythematous rash; in a few cases it was scarlatiniform or morbilliform. Thirty-eight of the 223 cases ended fatally; in a number of instances, death occurred within a few days after the administration of serum before sufficient time had elapsed for a rash to develop. In only 9 of these 38 cases was the occurrence of a rash noted. If these 38 cases are omitted, 185 cases are left, of which 149 showed a rash at some period; i.e. 80 per cent.

The occurrence of painful joints was noted in a considerably smaller number of cases, only in 68 of the 185 cases, and general glandular enlargement was noted in only 36 of the 185 cases. In some cases, however, enlarged glands, or painful joints, were noted where there was

no skin rash, so that among the 185 cases there occurred 153 in which skin rash, glandular enlargement, or painful joints were present at some period, namely, in 85.4 per cent of the cases.

When our former statements regarding the frequency of occurrence of serum sickness were made, we disregarded those cases in which urticaria occurred immediately following the administration of serum and those cases in which the signs and symptoms were hardly noticeable. Employing, however, your definition of serum disease, at least 85 per cent of our cases may be said to have suffered from this condition, and considering the fact that in a few instances we undoubtedly overlooked very minor manifestations, it is quite probable that the statement that "90 per cent of patients following administration of foreign serum suffer from serum disease" is correct.

Further support of Dr. Cole's opinion was obtained in a study of a series of 367 cases receiving intravenous injections of serum in the Boston City Hospital. We are indebted for permission to publish these statistics to Dr. Edwin H. Place, Physician in Chief to the South Department of the Boston City Hospital. Ninety-nine of this series were cases of pneumonia that had received anti-pneumococcus serum. We are indebted to Miss L. M. Corcoran for assistance in compiling the data on these ninety-nine cases. The average amount of serum injected in these cases was 272 cc. The incidence of serum disease recorded in the histories is 67.7 per cent.

The remaining 268 individuals were cases of diphtheria in the South Department of the hospital and received an average of 150 cc. of diphtheria antitoxin serum. As antitoxin is administered in this department sometimes in the form of the pseudoglobulin fraction and sometimes as whole serum, it was necessary for us to inquire in each instance as to the nature of the preparation used. This was readily done because the laboratory serial number of the preparation injected was always noted in the record. We have been able to obtain this information through the kindness of Dr. Benjamin White, Director of the Division of Biologic Laboratories of the Massachusetts Department of Public Health, in whose laboratories the antitoxin was prepared.

The incidence of serum disease in the diphtheria cases was 70 per cent. It should be noted that the incidence in this series receiving an average of 150 cc. is about the same as that of the pneumonia series receiving 272 cc. Fifty-six individuals received less than 100 cc. and of these fifty-six, eight received less than 50 cc.

These percentages are so close to the original estimated 70 per cent of Dr. Cole that they furnish strong support to his conclusion that some form of serum reaction follows the intravenous injection of horse serum in about 90 per cent of individuals so treated. The correction introduced by Dr. Cole in his original estimate is particularly applicable in the Boston series on account of the fact that all cases that were under observation for ten days or more are included in it.

The evidence presented above seems to leave little doubt as to the approximate correctness of the figure, 92.4 per cent, used in our comparison of the two races to indicate the incidence of serum disease in the white race. This being the case, the estimated ratio of susceptibility of the two races to serum disease may be regarded with some confidence as indicating, at least, a wide difference in the relative susceptibility.

It is seen that a similar difference in the relative susceptibility of the two races is exhibited in both allergy (hay-fever, asthma, urticaria) and serum disease. This fact by no means proves these two conditions to be due to the operation of an identical mechanism. It merely suggests a similarity of mechanism in both conditions, which may not be complete.

SUMMARY

Through inquiry it has been found that the American Indian is apparently much less frequently affected by the allergies than is the white race. An experimental study of the occurrence of serum disease in twenty-six volunteer full-blood American Indians indicates that the Indian race is much less susceptible to that condition than is the white race.

This similarity in the relative susceptibility of the two races to these two conditions suggests a similarity in the underlying mechanism of both of the conditions which, however, need not amount to a complete identity.

REFERENCES

1. Hrdlička Aleš, Smithsonian Institution, Bureau of American Ethnology. Bulletin 34, 1908.
2. Lain, E. S., Journ. Amer. Med. Assoc., July 19, 1913, page 168.

FRONT ROW

William Frank, Joe Bearhead, Benjamin Osage, Harber Johnson, William Atkins, Thomas Wasson, Delmar Scott, Robert Leve Leve, William Hampton, Lawton Raymond, Joseph Parnell

MIDDLE ROW

Francis Cornelius, Agnes Hildebrand, Hazel Dupuis, Lenna Dann, Nancy McKnight, Ida Primeaux, Miss Hannah Anderson, Dr. Edward F. Menger, Charles Hutchison, Andrew Juan, Edward Meeks

BACK ROW

Wallace Littlefinger, Edward Davenport, Emery Redbird, Morris Baker, William Ruskin, Marion Runsthru, Andy Snap, Jesse King, Mason Kawaykla, Walter Emarthla, James Foster, Abel Archibald

STUDIES IN SPECIFIC HYPERSENSITIVENESS

IX. ON THE PHENOMENON OF HYPOSENSITIZATION (THE CLINICALLY LESSENED SENSITIVENESS OF ALLERGY)

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In 1911, the writer began the application of the principle of desensitization to the treatment of the allergies (hay fever, asthma, urticaria, angio-neurotic edema).

This principle was employed under the influence of the view of Wolff-Eisner and of Meltzer that the hypersensitiveness of human beings is an expression of anaphylaxis; that is, dependent upon the presence in the sensitive tissue of specific antibodies. Coca (1, 2), in a recent analysis of the phenomena of anaphylaxis and those of allergy, has pointed out differences between these two conditions of such a nature as to make an identity of the two seem unlikely.

One of these differences is a fact that had been observed by the writer in innumerable instances; namely, that although a certain degree of lessened sensitiveness can be obtained in allergy by administration of the exciting agent, either in its natural state by mouth or in extracts by injection, this effect in human beings has never been observed to approach the entire insensitiveness of the condition of "complete desensitization" in anaphylaxis in the lower animals.

In illustration of this principle, the following concrete observations were drawn by Coca out of our unpublished records. In all of the individuals suffering from any form of allergy, who have been rendered clinically insensitive to the natural contact with the exciting agent, the suitable administration of the agent,

by intracutaneous or subcutaneous injection will demonstrate the persistence of the hypersensitiveness.

In some of our cases, indeed, it could be shown that even the insensitiveness to the natural contact was only relative. For example patient A. K., No. 1107,[1] sensitive to egg, was brought by appropriate injections and oral administration of increasing doses of egg white to the point where one whole egg every other day was tolerated, but on several trials he was found unable to eat two eggs on one day without exhibiting symptoms.

The phenomenon of desensitization is a constant characteristic of the condition of anaphylactic hypersensitiveness in all the lower animals in which the phenomenon has been studied. This must be true because the hypersensitiveness of anaphylaxis depends upon the presence of antiprotein antibodies (precipitins) in the tissues; and because it is always possible by a suitable manner of injection of the antigen to neutralize these precipitins so gradually as to avoid the physiological reaction of anaphylaxis and to neutralize them so completely that the hypersensitiveness is *entirely* removed.

It would seem, therefore, that desensitization, which is a constant characteristic of anaphylaxis, should be demonstrable in that condition in every animal in which anaphylaxis occurs. A conceivable exception might exist in an animal in which the antibody production was so rapid that the neutralized precipitins were immediately replaced. However, no such exception is known.

It would seem natural for one seeking to demonstrate desensitization in allergy to choose serum allergy as the first object of study; particularly, the ordinary form of this condition in which the clinical symptom of urticaria is only exhibited after an incubation period following the injection of serum and which is generally accompanied by precipitin formation. However, no one has yet applied the procedure of desensitization to individuals under these circumstances.

In the rarer form of serum allergy, in which symptoms appear

[1] This case is described in greater detail in the article on constitutional reactions; this Journal volume 7, p. 141.

immediately after a primary injection and in which the previous existence of antibodies has not been demonstrated (3), attempts have been made to reduce the sensitiveness by the injection of increasing quantities of serum, beginning with small doses.

In his paper on "Asthma Complicating the Serum Treatment of Pneumonia" (4), H. L. Alexander describes three cases in two of which the procedure just referred to was employed. In one of these the subcutaneous injection of 1 cc. caused urticaria, and the same quantity injected intravenously two and one-half hours later caused nausea, vomiting and asthma. One and a half hours after this occurrence 1 cc. was again injected and this again caused nausea and vomiting, but without an asthmatic attack. Two further injections of 2 cc. and 4 cc. produced no symptoms and a final intravenous injection of 65 cc. was accomplished with no more serious effect than an attack of "hay fever" and of asthma.

Another individual was brought gradually to the intravenous injection of 1 cc. of serum without symptoms. Then the intravenous injection of 40 cc. of the serum produced asthma and subsequent injections of 40 cc., 60 cc., 65 cc., 70 cc., and 75 cc. at from six to eight and a half hour intervals all caused mild or moderate "reactions." Alexander adopted the view, at that time unopposed, that he was dealing with a state of anaphylactic sensitiveness. He overlooked the difficulty attaching to this view by reason of the uniform absence of demonstrable antibodies in the form of serum sensitiveness that he was studying.

In a paper entitled "Serum Desensitization" (5), George M. Mackenzie added two further cases to those of Alexander. In one of these 1 cc. of serum injected intravenously produced respiratory difficulty and marked urticaria. An identical injection twelve hours later caused no symptoms, but after a further interval of one and a half hours, 2 cc. again produced marked urticaria, this time with edema. In the second case 16 cc. of serum produced eruption, although 8 cc. administered three quarters of an hour previously had caused no symptoms.

In all of these experiments the writer's experiences referred to above have been duplicated. A lessened sensitiveness was

attained in a practically important degree, but not complete *insensitiveness.* Moreover, this result was obtained under circumstances—the *repeated intravenous injection of relatively large quantities of serum*—which warrant the conclusion that complete specific insensitiveness is not attainable in allergy.

The uniform failure to induce complete insensitiveness in allergy indicates, according to Coca, that the relative insensitiveness attained in allergic conditions is of a nature quite different from that of desensitization. The objection may be raised against Coca's conclusion that the difference between these two effects need not be a qualitative one; it may be only quantitative. That is, the clinical insensitiveness attainable in allergy may represent a partial desensitization. In fact, a superficial examination favors this latter view.

However, facts are at hand to prove that the difference between the two effects is not quantitative but qualitative. They indicate, by the same token, that the allergic hypersensitiveness of human beings is not due to the influence of precipitin—it is not anaphylaxis.

We are referring to the results published by Coca and Kosakai (6) in their study of the reactivity of partially neutralized precipitin. These investigations showed that the partial neutralization of precipitin takes place according to a law which is quite different from that governing partial chemical precipitation. As they did not analyze their experiments from this point of view, we will do it here.

In their table (1) it appears that in guinea-pigs passively sensitized with 0.4 cc. of a precipitating serum, only slight symptoms were caused by the injection of 0.0025 cc. of the antigen solution. After a partial neutralization of the precipitating serum with a quantity of the antigen corresponding with 0.0008 cc. animals were sensitized with 0.4 cc. of the treated serum and it was found that in these animals 0.01 cc. of the antigen was required to produce slight symptoms; that is, twelve and a half times as much as was used for the partial neutralization.

In a similar experiment (table 4) in which the partial neutralization was carried out *in vivo* with 0.008 cc. of the antigen 0.1 cc. was required to cause symptoms in the sensitized guinea-

pigs—again twelve and a half times the quantity of antigen used for the partial neutralization of the antiserum (partial desensitization in this case).

A third experiment (table 8) with a different serum, resulted in a larger ratio between the dose of antigen used for partial desensitization and that required to produce symptoms. The ratio in 8a was 0.00025:0.0133::1:53; in 8b 0.0025:0.2::1:80. The discrepancy between these two ratios is due to experimental error.

In the same paper Coca and Kosakai were able to demonstrate that the reactivity of partially neutralized precipitin, as judged *in vitro* by the criterion of precipitation, is quantitatively the same as it is *in vivo* as judged by the criterion of anaphylactic shock.

The fundamental difference between the quantitative laws governing immunological and chemical precipitation is seen in a comparison of the experiments just described and the following: if to 10 cc. of a decinormal solution of silver nitrate 1 cc. of decinormal hydrochloric acid is added and if the resultant precipitate is removed a further precipitation can be produced by the second addition of 1 cc. of decinormal hydrochloric acid; moreover, the second precipitate will be of exactly the same weight as was the first.

The experiments of Coca and Kosakai reveal a simple but fundamentally characteristic peculiarity in the partial neutralization of precipitin which may be used as a test to determine the nature of human hypersensitiveness. If the latter is dependent upon the presence of precipitin, then the successive injection of identical or nearly identical quantities of the exciting agent should not cause repeated exhibition of symptoms. A multiple of the partially desensitizing dose is always required to cause symptoms upon a subsequent injection. Conversely, if symptoms, even in slight degree, recur upon such repeated injections, then precipitin can have no part in the production of the symptoms.

Applying this test to the observations of Alexander and of Mackenzie which we have referred to above, we must conclude that the serum sensitiveness which they were attempting to

modify was not anaphylactic in nature and that the reduced sensitiveness which they established was therefore not a partial desensitization.

In the first case cited from Alexander, two successive intravenous injections of 1 cc. of serum were followed in each instance by symptoms which were altered at the second injection but still marked.

In the second case six successive injections of nearly equal and large quantities of serum each produced symptoms.

In one of Mackenzie's cases an intravenous injection of 1 cc. of serum was given without symptoms, yet one and a half hours later 2 cc. caused marked reaction. The conditions in Mackenzie's second case were similar to those of the first and the results were the same.

One of the outstanding and practically important features of allergic hypersensitiveness is the specific reactivity of the skin. The easy accessibility of this tissue invited study of the effect of injections of the exciting agent of the hypersensitiveness upon the cutaneous reaction.

The writer had had numerous opportunities to make such a study following subcutaneous injections given in hay fever and asthma for therapeutic purpose. The effect of those injections has usually been a specific lessening of the general cutaneous reactivity, which, however, never approached extinction.

Recently Mackenzie and Baldwin (7, 8) have investigated the effect of local application of the exciting agent upon the cutaneous reaction and they have concluded that after repeated applications to the same site the reactivity of that site is specifically exhausted. In some instances the exhaustion did not persist longer than twenty-four hours; in one case, however, the intracutaneous injection of a 1:10 dilution of egg white caused a complete suppression of reactivity in the same site for three days.

In several unpublished experiments we had been unable to demonstrate an exhaustion of the cutaneous reactivity after an interval of about twenty hours following one or more intradermal injections, although in some instances the solution used for the attempted exhaustion was several times stronger than that used for the final test on the following day.

In further experiments, which we are about to describe, we have made repeated intradermal injections in the same site at short intervals and we have succeeded in producing practically complete exhaustion of the reactivity of the site to the substance in the particular concentration in which it had been injected. However this effect could be shown not to be specific; it appears, thus, to be merely a temporary exhaustion or fatigue of the general power of reactivity on the part of the tissues to irritation.

The experiments were carried out by the writer on himself. This circumstance permitted a continuous observation as to both the objective and the subjective phenomena during the considerable period of time occupied by the tests.

The writer is highly sensitive to horse and rabbit dandruff and also to the serum of these two animals. The first experiment was carried out with extracts of the danders; in the second experiment the two sera were used.

In both experiments the volume of fluid injected was always the same—0.01 to 0.02 cc. The concentration of the allergens in the different solutions is indicated by the quantity of nitrogen in 1 cc. of fluid as determined with the Kjeldahl method. For example, horse epithelium 0.01 means an extract of horse dander 1 cc. of which contained 0.01 milligram of nitrogen.

All injections were made intradermally and when repeated injections were made in the same site the needle was always introduced into the same puncture orifice.

EXPERIMENT 1[2]

July 23.

12:35 p.m. Horse epithelium 0.01 is injected into site 1 on the anterior surface of the left forearm. 12:50 p.m. The area of reaction is drawn (fig. 1, chart 1.

4:55 p.m. The wheal at site 1 has disappeared but the site is still red and there is an area of edema of the size of a quarter. Horse epithelium 0.01 is injected into site 1.

[2] On July 25 at the time the tests were made with horse epithelial extract 0.05 milligram of nitrogen per cubic centimeter there were noted some constitutional effects—coryza, asthma and slight urticaria. About five days after these tests were made there developed further mild symptoms of asthma with coughing and

5:05 p.m. The area of reaction is drawn (fig. 2, chart 1). The wheal is somewhat larger than that produced by the first injection and there is marked itching as at first.

10:15 p.m. Site 1 still presents an area of redness and edema measuring about 2 by 3 inches. Horse epithelium 0.01 is injected into site 1.

10:30 p.m. The area of reaction is drawn (fig. 3, chart 1). There is no increase in the zone of hyperemia. A wheal is again formed accompanied by considerable itching.

July 24

8:15 a.m. The zone of redness and edema still persists (2 by 3 inches). Horse epithelium 0.01 is injected into site 1.

8:30 a.m. The area of reaction is drawn (fig. 4, chart 1). The reaction as a whole is distinctly less than at the first injection.[3]

2:10 p.m. There is very slight edema and no redness at site 1. Horse epithelium 0.01 is injected into site 1.

2:20 p.m. The area of reaction is drawn (fig. 5, chart 1). The wheal is of about the size of the earlier ones but it is of shorter duration; the zone of hyperemia is strikingly decreased.

4:10 p.m. There is practically no edema and no redness at site 1. Horse epithelium 0.01 is injected into site 1.

4:25 p.m. The area of reaction is drawn (fig. 6, chart 1). The wheal is less sharply defined and hyperemia is almost absent.

6:20 p.m. Horse epithelium 0.01 is injected at site 1.

6:30 p.m. The area of reaction is drawn (fig. 7, chart 1). The wheal is ill defined, merging into a small zone of hyperemia.

10:30 p.m. Horse epithelium 0.01 is injected into site 1.

10:45 p.m. The area of reaction is drawn (fig. 8, chart 1). The reaction is distinctly more marked than it was at the last preceding injection. There is no itching.

expectoration and an occasional hive. This necessitated the use of adrenalin in a dose of ten minims two to three times daily. The condition lasted for ten days and then subsided over a period of four days. After the second series of tests with serum to which the writer is less sensitive there was no constitutional reaction aside from an occasional hive during four to five days after the last test.

[3] It must be borne in mind that the relative duration of the reactions and the relative intensity of the hyperemia are not indicated in the drawings.

July 25

7:30 a.m. There is a small papule ¼ inch in diameter about the puncture orifice. The papule is slightly tender and not red. Horse epithelium 0.01 is injected into site 1.

7:45 a.m. The area of reaction is drawn (fig. 9, chart 1). The reaction is of short duration. There is no itching.

9:15 a.m. The papule is slightly tender; there is no redness. Horse epithelium 0.05 is injected into site 1.

9:30 a.m. The area of reaction is drawn (fig. 10, chart 1). The wheal is larger than the preceding one; there is no hyperemia and no itching. The wheal is of short duration.

10:40 a.m. The papule is tender; there is no redness. Horse epithelium 0.1 is injected into site 1.

10:50 a.m. The area of reaction is drawn (fig. 11, chart 1). The injection of this more concentrated solution caused an evanescent hyperemia and considerable itching which was felt also in the cubita fossa.

5:55 p.m. Only the papule persists. Horse epithelium 0.1 is injected into site 1 and also into another place on the same forearm—site 2.

6:05 p.m. The two areas of reaction are drawn (fig. 12a, chart 1—site 1; fig. 12b, chart 1—site 2). The reaction at site 1 was of very short duration without itching. That at site 2 was accompanied with itching, hyperemia and edema which persisted until 6:00 p. m. on the following day.

6:15 p.m. Rabbit epithelium 0.05 is injected into the lower anterior surface of the right forearm—site 3.

6:30 p.m. The area of reaction at site 3 is drawn (fig. 13a, chart 1). There is a lymphangitis which is visible at intervals from the injection site to the cubital fossa, where it is especially marked.

8:30 p.m. The area of reaction at site 3 is again drawn (fig. 13b, chart 1). Both the wheal and the zone of hyperemia have increased. On the following morning edema and redness were still present.

8:35 p.m. Rabbit epithelium 0.5 is injected into site 1.

8:50 p.m. The area of reaction of site 1 is drawn (fig. 14, chart 1). There is a slight lymphangitis extending to the cubital

fossa and a very slight lymphangitis from the fossa to the axilla.

10:45 p.m. The reaction produced at site 1 by the last injection has subsided; no trace of it is left.

July 26

4:00 p.m. Horse epithelium 0.1 is injected into site 1.

4:15 p.m. The area of reaction at site 1 is drawn (fig. 15, chart 1). The resulting wheal was larger than that of 12a. It persisted for one-half hour and was accompanied with itching, which extended up through the cubital fossa into the axilla.

EXPERIMENT 2

August 25

8:45 p.m. Horse serum 0.1 is injected into site a on the right forearm

9:00 p.m. The area of reaction is drawn (fig. 1a, chart 2).

8:50 p.m. At site x on the same forearm rabbit serum 1 was injected as a preliminary test of the cutaneous reactivity to that material.

9:05 p. m. The area of reaction at site x is drawn (fig. 1b, chart 2).

August 26

7:45 a.m. Site a is still red and slightly edematous. Horse serum 0.1 is injected into site a.

8:00 a.m. The area of reaction is drawn (fig. 2, chart 2). The reaction appears to be slightly greater than after the preceding injection. This is doubtless due to the persistence of the effects of the first injection.

12:45 p.m. Some of the wheal and of the hyperemia still persist from the second injection. Horse serum 0.1 is injected into site a.

1:00 p.m. The area of reaction is drawn (fig. 3, chart 2).

8:25 p.m. There is still some hyperemia at site a. Horse serum 0.1 is injected into site a.

8:35 p.m. The area of reaction at site a is drawn (fig. 4, chart 2). There is some itching in the site, but no increase in the hyperemia and little in the wheal.

11:30 p.m. Slight hyperemia still persists at site a. Horse serum 0.1 is injected into site a.

11:40 p.m. The area of reaction is drawn (fig. 5, chart 2). There is slight itching in the site but no real increase in the wheal nor in the hyperemia.

August 27

7:45 a.m. Horse serum 0.1 is injected into site a.

8:00 a.m. The area of reaction at site a is drawn (fig. 6, chart 2). There is very slight itching and faint hyperemia in the site. The wheal is not increased in size. There is an urticarial spot on the right thigh.

9:00 a.m. Horse serum 1 is injected into site a.

9:10 a.m. The area of reaction at site a is drawn (fig. 7, chart 2). This reaction lasted only twenty minutes. There was a constitutional reaction—asthma, with generalized itching and coryza with itching of the eyes—which persisted for about two hours.

12:10 p.m. Horse serum 1 is injected into site a and into site b on the left forearm.

12:20 p.m. The area of reaction at site a is drawn (fig. 8a, chart 2). This reaction lasted fifteen minutes. There was a constitutional reaction with coryza and generalized itching which lasted about two hours. Five urticarial spots appeared on the body.

12:25 p.m. The reaction at site b is drawn (fig. 8b, chart 2). This reaction persisted for twenty-four hours.

3:00 p.m. Rabbit serum 1 is injected into site a and into site c on the left forearm.

3:10 p.m. The area of reaction at site a is drawn (fig. 9a, chart 2). There is no itching and no definite hyperemia. The size of the wheal is not increased. The reaction at site is marked, though not so intense as that at site b.

3:15 p.m. The area of reaction at site c is drawn (fig. 9b, chart 2). The reaction persisted for about twenty hours, with edema, itching and hyperemia.

Both of these experiments bear out the conclusions which we arrived at in our discussion of the observations of Alexander and of Mackenzie: namely, that there is no true desensitization in allergy. In experiment 1, three successive injections of the same quantity of horse epithelium 0.01 produced marked reactions

(figs. 1, 2, and 3, chart 1). In experiment 2, two successive injections of horse serum 0.1 produced marked reactions.

The second noteworthy feature of both experiments is that after a site has been made nearly insensitive to one concentration of the material that is being injected, a vigorous reaction can be produced with a stronger concentration of the same material (fig. 11, chart 1; and fig. 7, chart 2).

In this fact may be seen a possible explanation of the belief of Mackenzie and Baldwin that the local "exhaustion" of the allergic reaction is specific. These authors may have tested the exhausted site with a second protein in a concentration higher than that of the protein used for the exhaustion.

The third result of the tests, which is evident in both experiments, is that the local insensitiveness produced by the repeated injections is not specific, as Mackenzie and Baldwin thought. It is merely the well known nonspecific fatigue of the tissues to protracted irritation.

Before this condition of fatigue has set in, the allergic mechanism continues to function upon repeated identical injections and even after the tissues have become fatigued to one concentration of the allergen the mechanism can be shown to be still intact by the injection of the allergen in greater concentration.

In experiment 1, after site (1) had been rendered insensitive to horse epithelium 0.1, the injection of the stronger extract of rabbit epithelium 0.5 produced a distinct reaction. This reaction was, however, much weaker in intensity and duration than that caused by the same injection into the fresh site (3). It was probably no greater than one that would have been produced in the "exhaused" site by horse epithelium 0.5.

In experiment 2, the drawings of the final reactions are in themselves convincing evidence of the non-specificity of the local insensitiveness produced by repeated injections.

The necessity of seeking further evidence to support the foregoing interpretation of our experiments has been recognized. It was necessary, first, to find out whether a non-specific local exhaustion of the skin is possible and, secondly, if this was true, to see whether any significant difference is demonstrable in the

development of this non-specific local exhaustion and of that induced by the repeated injection of allergens in specifically sensitive individuals. These two questions were investigated in some experiments that were carried out in this department by Dr. W. C. Spain and Dr. Ruth Guy with peptone and histamin.

The susceptibility of the skin to peptone was first described by Philippson in the Giorn. ital. d. mal. ven. e. d. pelle in 1899; that to histamin was first described by Eppinger in the Wien. med. Woch. in 1913. Since the local effect of both of these substances is non-specific they lent themselves well to the purpose in mind.

Mackenzie and Baldwin (8) state that Sollman found the histamin skin reaction inexhaustible and that they themselves found this reaction actually to increase in size and intensity with each successive application of the substance. A review of the experiments of Sollman (9, 10) shows that they are inadequate to determine this question. In the earlier experiments with Pilcher (9) he states (page 313), "When repeated applications are made (histamin) whether to the same or to the opposite arm, the wheals of the later applications appear smaller than the earlier." In his later study (10) Sollman describes only one experiment (no. 18) in which a "repeated" application of histamin was made at one site. In this experiment the second application of a $\frac{1}{10}$ per cent solution after a thirty-five minute interval produced a more vigorous reaction than had the first. No further applications were recorded. Evidently no information regarding the possible exhaustibility of the histamin reaction is obtainable from this experiment.

Mackenzie and Baldwin (8), also, used the strong $\frac{1}{10}$ per cent solution of histamin. They do not state how many applications were made nor what intervals of time were observed. In the experiments by Dr. Spain and Dr. Guy the concentration of the two substances in the solutions is given in percentage of the dry material. The quantity injected was the same as in the writer's experiment (0.01 cc.). All of the injections were intradermal. In the first experiment by Dr. Spain all of the injections were made into the same puncture orifice on the left forearm as follows:

July 23

12:55 p.m. Histamin, 0.01.[4]
1:15 p.m. Area of reaction drawn (figure 1, chart 3).
2:30 p.m. Histamin, 0.02.
2:45 p.m. Area of reaction drawn (figure 2, chart 3).
3:00 p.m. Histamin, 0.04.
3:20 p.m. Area of reaction drawn (figure 3, chart 3). The outlines of the wheal are indistinct.
4:00 p.m. Histamin, 0.04.
4:20 p.m. Area of reaction drawn (fig. 4, chart 3). The wheal forms slowly and is indistinct.
4:50 p.m. Histamin, 0.04.
5:10 p.m. Area of reaction drawn (fig. 5, chart 3). The wheal is not elevated above the level of the surrounding tissue; its outline is indistinct.
7:15 p.m. Histamin, 0.04.
7:35 p.m. Area of reaction drawn (fig. 6, chart 3). The outline of the wheal is questionable.
8:10 p.m. Histamin, 0.04.
8:30 p.m. Area of reaction drawn (fig. 7, chart 3). The outlines of the reaction are very indistinct: the surrounding skin is indurated in a space about 3 inches in diameter. There is no definite wheal.
8:30 p.m. Histamin, 0.2. No definite wheal resulted from this injection. No drawing was made.

July 24

8:30 a.m. The area of induration still persists, with a shot-like central node. No injection made at this time.
11:45 a.m. Histamin, 0.4.
12:00 a.m. Area of reaction drawn (fig. 8, chart 3). A very indistinct wheal is formed which is only slightly elevated. There is little erythema.

July 26

4:00 p.m. Histamin, 0.4.
4:30 p.m. The outlines of the reaction are very indistinct Area of reaction drawn (fig. 9, chart 3).

[4] 100 cc. of this solution contained 0.01 grams of histamin. The substance was obtained from Burroughs Wellcome & Co. under the name Ergamine Acid Phosphate.

In the second experiment by Dr. Spain, all of the injections excepting the final control injection were made into the same puncture orifice on the left forearm as follows:

September 13

9:00 a.m. Histamin 0.1.

9:15 a.m. Area of reaction drawn (fig. 1, chart 4). There is decided erythema and much itching after five minutes.

10:00 a.m. Histamin, 0.1. The reaction caused by the first injection has subsided. There is immediate itching.

10:05 a.m. There is a sensation of dizziness and "light headedness." which lasts about one to two minutes. Blood pressure is 112/80. (The normal pressure is 118/80 –116/80.)

10:10 a.m. Area of reaction drawn (fig. 2, chart 4)

11:00 a.m. Histamin, 0.1. The wheal and most of the erythema have disappeared.

11:15 a.m. Area of reaction drawn (fig. 3, chart 4). The reaction is apparently equal to the previous one.

12:05 p.m. Histamin 0.1.

12:15 p.m. Area of reaction drawn (fig. 4, chart 4). There is very little reaction; the size of the wheal and extent of the erythema are not increased.

2:00 p.m. Histamin, 0.1.

2:15 p.m. Area of reaction drawn (fig. 5, chart 4). There is little erythema and little increase in the size of the wheal.

3:45 p.m. Histamin 0.2.

3:55 p.m. Area of reaction drawn (fig. 6, chart 4). There is some increase in the size of the wheal, though the reaction is not violent. The zone of erythema is comparatively small.

5:00 p.m. Histamin, 0.2.

5:10 p.m. Area of reaction drawn (fig. 7, chart 4). The reaction was slight.

September 14

9:00 a.m. Histamin 0.2.

9:15 a.m. Area of reaction drawn (fig. 8, chart 4). The reaction was slight; the hyperemia was hardly visible.

9:30 a.m. Histamin, 0.2.

9:45 a.m. Area of reaction drawn (fig. 9, chart 4). There is no increase in the size of the wheal; the hyperemia is vague.

11:00 a.m. Histamin, 0.4.
11:15 a.m. Area of reaction drawn (fig. 10, chart 4). The wheal remains the same and the hyperemia is very slight.
11:00 a.m. Histamin 0.4 in right forearm.
11:15 a.m. Area of reaction drawn (fig. 11, chart 4).

The experiment by Dr. Guy was carried out on herself as follows:

September 22

10:29 a.m. Histamin, 0.02, site 1.
10:37 a.m. Area of reaction drawn (fig. 1, chart 5)
11:55 a.m. Histamin, 0.02, site 1.
12:07 p.m. Area of reaction drawn (fig. 2, chart 5).
2:07 p.m. Histamin, 0.02 site 1.
2:40 p.m. Area of reaction drawn (fig. 3, chart 5.) The wheal was rather indefinite.
4:00 p.m. Histamin, 0.02, site 1.
4:12 p.m. Area of reaction drawn (fig. 4, chart 5). Wheal again not sharply defined.

September 24

11:11 a.m. Histamin, 0.02, site 1.
11:16 a.m. Area of reaction drawn (fig. 5, chart 5). A definite reaction.
12:50 p.m. Histamin, 0.02, site 1.
1:04 p.m. Area of reaction drawn (fig. 6, chart 5). The reaction was slight.
2:09 p.m. Histamin, 0.02, site 1.
2:25 p.m. Area of reaction drawn (fig. 7, chart 5). Slight reaction.
3:56 p.m. Histamin, 0.02, site 1.
4:01 p.m. Area of reaction drawn (fig. 8, chart 5). Slight reaction.
9:08 p.m. Histamin 0.02, site 1 right forearm.
9:15 p.m. Area of reaction drawn (fig. 9, chart 5). Slight reaction.
11:03 p.m. Histamin 0.02, site 1.
11:17 p.m. Area of reaction drawn (fig. 10, chart 5). Slight reaction.

September 24

10:47 a.m. Histamin 0.02, site 1.
10:56 a.m. Area of reaction drawn (fig. 11, chart 5). Very slight reaction.

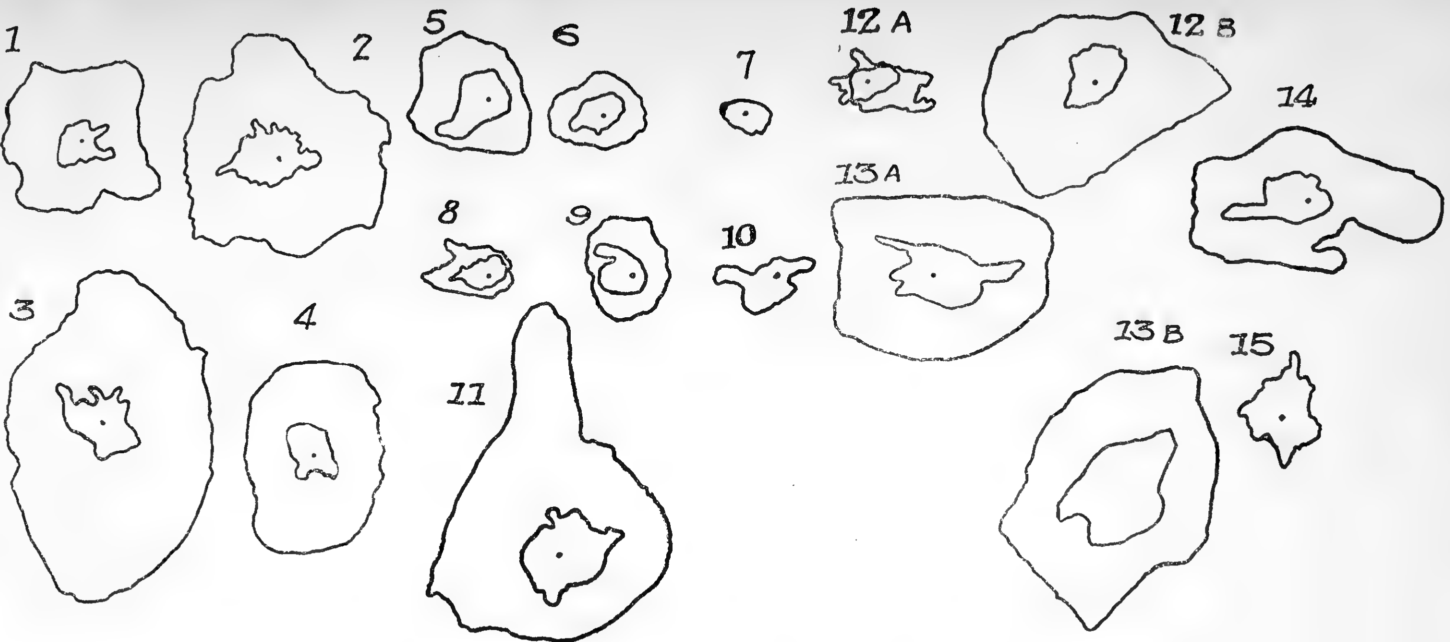

CHART 1

Fig. 1. Site 1; first injection; horse epithelium, 0.01.
Fig. 2. Site 1; second injection; horse epithelium, 0.01.
Fig. 3. Site 1; third injection; horse epithelium, 0.01.
Fig. 4. Site 1; fourth injection; horse epithelium, 0.01.
Fig. 5. Site 1; fifth injection; horse epithelium, 0.01.
Fig. 6. Site 1; sixth injection; horse epithelium, 0.01.
Fig. 7. Site 1; seventh injection; horse epithelium, 0.01.
Fig. 8. Site 1; eighth injection; horse epithelium, 0.01.
Fig. 9 Site 1; ninth injection; horse epithelium, 0.01.
Fig. 10. Site 1; tenth injection; horse epithelium, 0.05.
Fig. 11. Site 1; eleventh injection; horse epithelium, 0.1.
Fig. 12a. Site 1; twelfth injection; horse epithelium, 0.1.
Fig. 12b. Site 2; first injection; horse epithelium, 0.1.
Fig. 13a. Site 3; first injection; rabbit epithelium, 0.5.
Fig. 13b. Site 3; same reaction as in 13a, two hours later.
Fig. 14. Site 1; thirteenth injection; rabbit epithelium, 0.5
Fig. 15. Site 1; fourteenth injection; horse epithelium, 0.1.

The diameters of these drawings are exactly half those of the corresponding lesions.

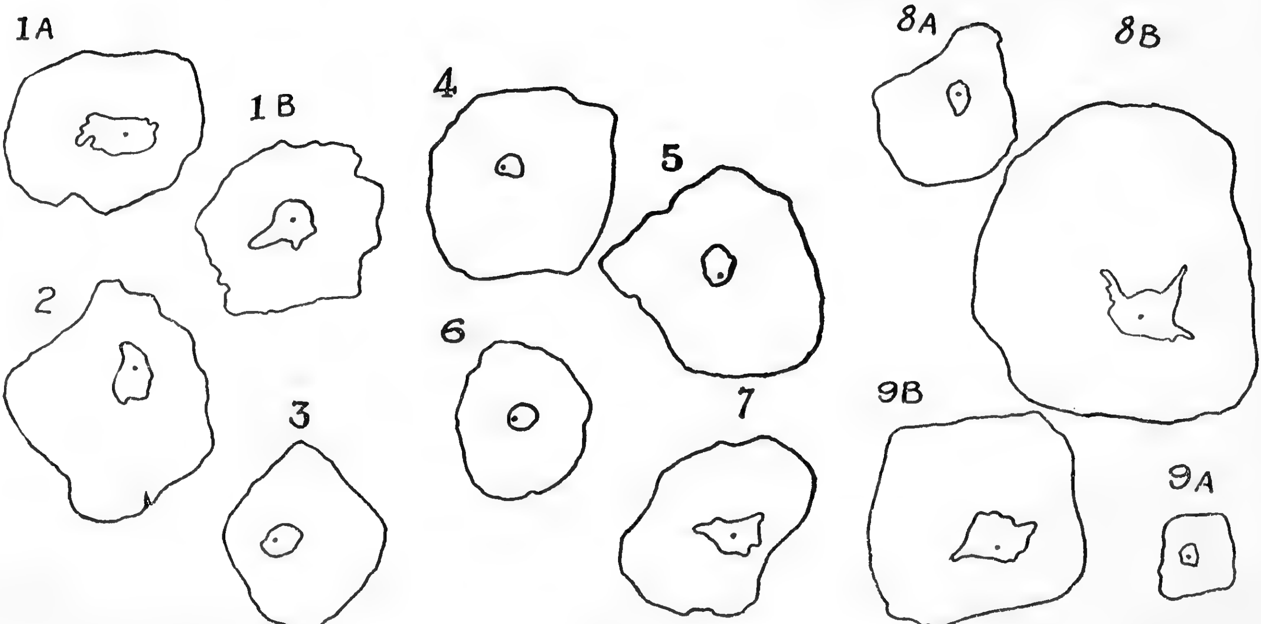

CHART 2

Fig. 1a. Site a; first injection; horse serum, 0.1.
Fig. 1b. Site x; first injection; rabbit serum, 1.
Fig. 2. Site a; second injection; horse serum, 0.1.
Fig. 3. Site a; third injection; horse serum, 0.1.
Fig. 4. Site a; fourth injection; horse serum, 0.1.
Fig. 5. Site a; fifth injection; horse serum, 0.1.
Fig. 6. Site a; sixth injection; horse serum, 0.1.
Fig. 7. Site a; seventh injection; horse serum, 1.
Fig. 8a. Site a; eighth injection; horse serum, 1.
Fig. 8b. Site b; first injection; horse serum, 1.
Fig. 9a. Site a; ninth injection; rabbit serum, 1.
Fig. 9b. Site c; first injection; rabbit serum, 1

The diameters of these drawings are exactly half those of the corresponding lesions.

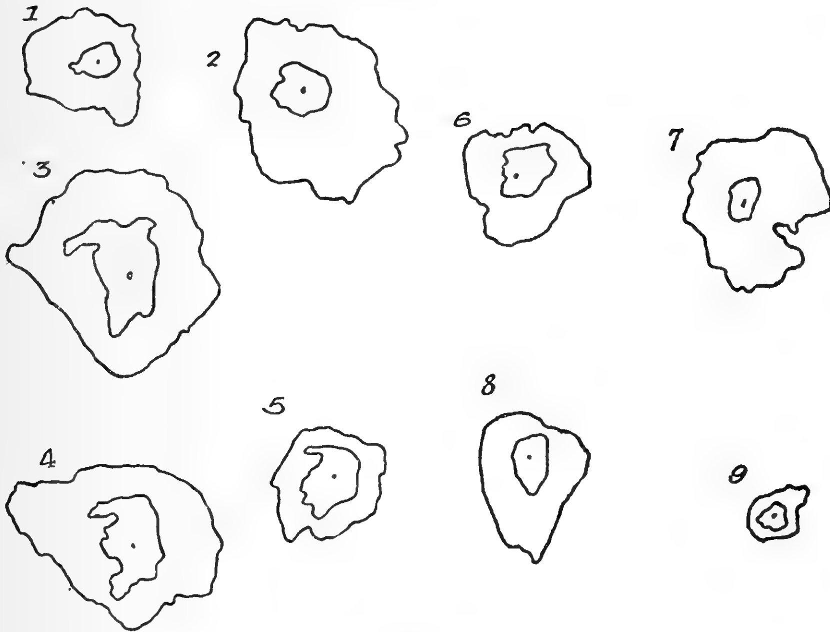

CHART 3

Fig. 1. Site a; first injection; histamin, 0.01 per cent.
Fig. 2. Site a; second injection; histamin, 0.02 per cent.
Fig. 3. Site a; third injection; histamin, 0.04 per cent.
Fig. 4. Site a; fourth injection; histamin, 0.04 per cent.
Fig. 5. Site a; fifth injection; histamin, 0.04 per cent.
Fig. 6. Site a; sixth injection; histamin, 0.04 per cent.
Fig. 7. Site a; seventh injection; histamin, 0.04 per cent.
Fig. 8. Site a; ninth injection; histamin, 0.4 per cent.
Fig. 9. Site a; tenth injection; histamin, 0.4 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.

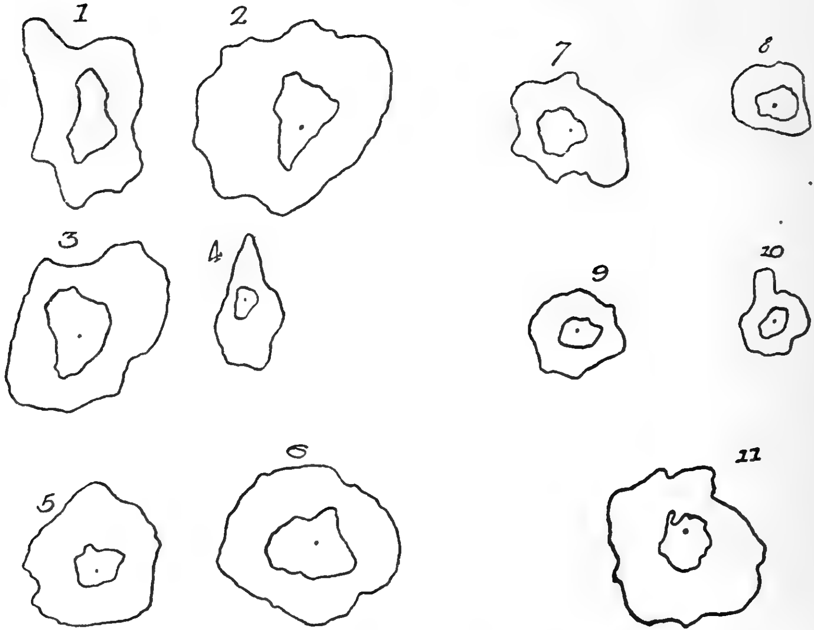

CHART 4

Fig. 1. Site a; first injection; histamin, 0.1 per cent.
Fig. 2. Site a; second injection; histamin, 0.1 per cent.
Fig. 3. Site a; third injection: histamin, 0.1 per cent.
Fig. 4. Site a; fourth injection; histamin, 0.1 per cent.
Fig. 5. Site a; fifth injection; histamin, 0.1 per cent.
Fig. 6. Site a; sixth injection; histamin, 0.2 per cent.
Fig. 7. Site a; seventh injection; histamin, 0.2 per cent.
Fig. 8. Site a; eighth injection; histamin, 0.2 per cent.
Fig. 9. Site a; ninth injection; histamin, 0.2 per cent.
Fig. 10. Site a; tenth injection; histamin, 0.4 per cent.
Fig. 11. Site b; first injection; histamin; 0.4 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.

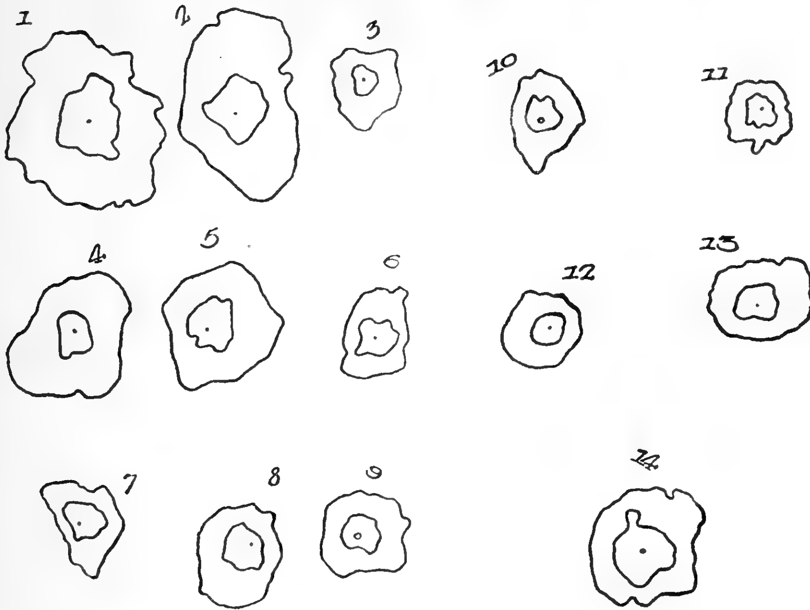

CHART 5

Fig. 1. Site 1; first injection; histamin, 0.02 per cent.
Fig. 2. Site 1; second injection; histamin, 0.02 per cent.
Fig. 3. Site 1; third injection; histamin, 0.02 per cent.
Fig. 1. Site 1; fourth injection; histamin, 0.02 per cent.
Fig. 5. Site 1; fifth injection; histamin, 0.02 per cent.
Fig. 6. Site 1; sixth injection; histamin, 0.02 per cent.
Fig. 7. Site 1; seventh injection; histamin, 0.02 per cent.
Fig. 8. Site 1; eighth injection; histamin, 0.02 per cent.
Fig. 9. Site 1; ninth injection; histamin, 0.02 per cent.
Fig. 10. Site 1; tenth injection; histamin, 0.02 per cent.
Fig. 11. Site 1; eleventh injection; histamin, 0.02 per cent.
Fig. 12. Site 1; twelfth injection; histamin, 0.02 per cent.
Fig. 13. Site 1; thirteenth injection; histamin, 0.2 per cent.
Fig. 14. Site 2; first injection; peptone, 5 per cent.

The diameters of these drawings are exactly half those of the corresponding lesions.

11:47 a.m.	Histamin 0.02, site 1. Area of reaction drawn (fig. 12, chart 5). Questionable reaction.
12:22 p.m.	Histamin 0.2 Site 1.
12:30 p.m.	Area of reaction drawn (fig. 13, chart 5). Questionable reaction.
12:45 p.m.	Peptone (Witte) 5.0, site 1.
12:55 p.m.	No reaction. No drawing was made.
12:45 p.m.	Peptone (Witte) 5.0, site 2, left forearm.
12:55 p.m.	Area of reaction at site 2 drawn (fig. 14, chart 5).

The materials used in the foregoing experiments by Dr. Spain and Dr. Guy produce their effect upon the skin by direct action and not by the mediation of an immunological reaction. This is evident from the fact that most individuals are susceptible to these substances upon the first administration of them. There are quantitative differences in this susceptibility which we may discuss in a future paper, but these differences do not contradict the principle just stated.

If the effect of the injection of the proteins of horse and rabbit dandruff is indirect—due to an immunological reaction—it should be expected that the existence of the intermediate immunological mechanism would be expressed in some difference in the results of the repeated injections as compared with those of the repeated injections of the directly acting histamin and peptone. However, a comparison of the five series shows them to be identical. In all, there is either an initial increase in the degree of the reaction or at least no change in it followed, then, by a decrease which is non-specific.

The evidence which we have presented seems to warrant the conclusion that the lessened sensitiveness induced in allergy by the injection of the exciting agent is a phenomenon which differs in its mechanism from that of desensitization in anaphylaxis. The former seems not to depend on the neutralization of precipitin as does the latter.

There is, however, to be considered the hypothetical possibility that the reactions of allergy depend on the presence in the tissues of "antibody-like" substances or so-called natural antibodies. This explanation was offered by Cooke, Flood and Coca (3)

but it was later dismissed by Coca (1), presumably on the ground that those hypothetical antibodies could not be neutralized completely. We hold this ground to be for the present a valid one because there is no known natural antibody which cannot be completely neutralized. It is useless to assume in allergy the existence of a natural antibody lacking a cardinal character of the known antibodies. One may as well assume the intermediary action of natural antibodies to explain the specific physiological effects of drugs.

On account of the confusion that must result from the use of the well defined term "desensitization" to designate clinically lessened sensitiveness in allergy it is suggested that the latter be referred to as a "hyposensitization." The etymological defect and also the convenience of this proposed term are the same as those of hypersensitization, which is in general use.

SUMMARY

1. The quantitative relations in partial desensitization are contrasted with those that obtain in the state of lessened sensitiveness in allergy, the differences being such as to indicate a difference in the mechanism of these two phenomena.

2. The phenomenon of "local exhaustion" of the allergic cutaneous reaction described by Mackenzie and Baldwin is studied and found, in disagreement with these authors, to be non-specific.

3. It is proposed to distinguish the lessened sensitiveness induced in allergy from the state of desensitization in anaphylaxis by designating the former condition as a state of hyposensitization.

REFERENCES

(1) Coca, A. F.: Hypersensitiveness. Tice's Practice of Medicine. W. F. Prior Company, Hagerstown, Md., vol. 1, p. 107.
(2) Coca, A. F.: Jour. Immunol., 1920, **5**, 363.
(3) Cooke, R. A., Flood, and Coca, A. F.: Jour., Immunol., 1917, **2**, 217.
(4) Alexander, H. L.: Arch. Int. Med., 1917, **20**, 636.
(5) Mackenzie, G. M.: Jour. Amer. Med. Assoc., 1921, p. 1563.
(6) Coca, A. F., and Kosakai, M.: Jour. Immunol., 1920, **5**, 297.
(7) Mackenzie, G. M., and Baldwin, L. B.: Proc. Soc. f. Exp. Biol. and Med., 1921, **18**, 214.
(8) Mackenzie, G. M., and Baldwin, L. B.: Arch. Int. Med., 1921, 722.
(9) Sollman and Pilcher.: Jour. Pharm. and Exp. Ther., 1917, **9**, 309.
(10) Sollman, T.: Jour. Pharm. and Exp. Ther., 1917–18, **10**, 147.

IMMUNOLOGICAL STUDIES ON TYPES OF DIPHTHERIA BACILLI[1]

I. AGGLUTINATION CHARACTERISTICS

II. PROTECTIVE VALUE OF THE STANDARD MONOVALENT ANTITOXIN

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The subject of this communication would have been exhaustively investigated long ago if there had been practical evidence that a monovalent diphtheria antitoxin did not protect from diphtheria. It is true that in regard to *B. tetani* and *B. botulinus* laboratory investigations have given contradictory results as to the efficacy of a monovalent serum. These investigations have shown us: first, that tetanus bacilli, although they are divided into several types by their response to agglutinins, nevertheless, all produce toxins which are neutralized by a monovalent antitoxin; second, that the reverse is true of the bacilli causing botulism, in so far as the production of toxin is concerned. Among the strains of *B. botulinus* there are at least two different types producing toxins which respond only to homologous antitoxins.

As to diphtheria bacilli, however, there are two series of observations which have been made concerning the degree of pro-

[1] Read at the meeting of the American Association of Immunologists, March, 1921.

This article combines the results of portions of two investigations. The first portion was a part of our studies on the respiratory infections and was designed to show the relative values of the absorption-agglutination method and other serological tests in determining epidemic strains. The second portion was a part of a series of studies on the similarity of the toxins produced by the diphtheria bacilli and the adequacy of a monovalent antitoxin.

In the studies on the absorption-agglutination characteristics of the diphtheria bacilli, we were given valuable aid by technicians supplied by the Influenza Commission of the Metropolitan Life Insurance Company.

tection afforded by monovalent diphtheria antitoxin. These observations have seemed to give all-sufficient evidence that the toxins produced by the different strains of the diphtheria bacilli are alike, at least, in their affinity for antitoxin. The first is, that for the last twenty-five years, ever since diphtheria antitoxin has been used in practice, injections of a monovalent antitoxin in hundreds of thousands of persons known to be in contact with infection, have given practically complete protection for two weeks—the period during which antitoxin is known to remain in appreciable amounts in a human being after injection. The second series of observations is that routine virulence tests throughout the world have with few, if any, exceptions shown that a monovalent diphtheria antitoxin is able to protect animals given a dose of culture fatal in others not given antitoxin.

If any doubt has existed in regard to the universal worth of a monovalent diphtheria antitoxin, it is one that has led to experimental investigation from time to time as to whether an antitoxic serum alone was as efficient as a combined antibacterial and antitoxic serum and whether from this standpoint a polyvalent serum might not be more advantageous. But the paper published by Havens (1) in 1920 seemed to show that diphtheria bacilli were divided into two agglutinative groups and that each group produced a toxin that was quantitatively at least, a very different one from that produced by the other group, so that a monovalent serum for group I had no appreciable effect on group II toxin unless the serum was given in enormous amounts. These findings, if correct, are of so much practical importance that reference will be made to them in some detail in connection with our own work.

I. AGGLUTINATIVE TYPES OF B. DIPHTHERIA

A number of investigators have reported, the obtaining of an agglutinating serum that differentiated between "true" and "pseudo" diphtheria bacilli (Schworer (2), Sander (3), Mason (4)), but no one had described definite agglutinative types among the toxic strains until Durand (5) published his observations in

1918. Durand announced that he had obtained four agglutinative types. In 1920 he published a further report in which he stated that on examining 252 strains by this test he found that they were divided as follows:

Type A contained	16 strains
Type B contained	8 strains
Type C contained	31 strains
Type D contained	76 strains
Type E contained	51 strains
Unclassified	71 strains

These types were strictly specific in that each absorbed only the respective agglutinins from the homologous serum.

Early in 1920 Dr. Durand visited our laboratory, demonstrated his methods, presented us with his type strains which he now designated with figures instead of letters and gave us the following facts in regard to their sugar fermentations: All ferment dextrose and levulose.

	MALTOSE	DEXTRIN	GLYCEROL	GALACTOSE	SACCHAROSE
Type I—Americaine ("our No. 8")	+	+	−	−	−
Type II—Durand	−	−	−	−	−
Type III—Nodet	+	+	+	+	−
Type IV—Benjamin	+	+	+	+	−
Type V—Sirbeaux	+	+	+	+	−

He also gave figures to support the following observation: There was less paralysis and attendant mortality in cases of diphtheria treated with diphtheria serum produced by the inoculation of both toxin and bacilli; i.e., an antitoxic and "antibacterial" serum, than with serum produced by the use of toxin alone. As Durand used all of the type strains in making this bacterial serum he had no evidence as to the effect of a monovalent antibacterial serum.

Our own work on agglutination. We immediately began inoculating a series of horses[2] with Durand's type strains and with our own strain of "no. 8," as well as with several strains from each

[2] This part of the work was done by C. R. Tyler and D. W. Poor.

of two apparent epidemics[3] of diphtheria, one in an institution in New York City and the other in a village near the City.

The horses were inoculated intravenously every day for three day periods, with suspensions of cultures prepared as follows:

The bacteria were grown for eighteen hours on Loeffler's coagulated blood serum medium at 37°C. The growths were then scraped into 0.85 per cent salt solution and transferred to tared Purdy tubes. They were sedimented in the centrifuge for twenty minutes and the supernatant liquid was removed. The weight of moist sediment was determined and the sediment was suspended in 10 cc. salt solution. The required weight of sediment (in suspension) was measured off and made up to 15 cc. for injection.

TABLE 1

Direct agglutination of the strains of B. diphtheriae used for toxin-antitoxin comparison

STRAINS	HORSE SERUM TITER					
	No. 8	Nodet	Sirbeaux	Benjamin	11	3362
Type I. Our "no. 8"	1600	0	0	0	0	
Type I. Americaine	1600	0	0	0	0	
Type III. Nodet	0	1600	0	0	0	0
Type IV. Benjamin	0	0	0	800	800	
Type IV. 11	0	0	0	800	800	
Type V. Sirbeaux	0	0	400	0	0	0
No. 3362	0	0	0	0	0	400

The first dose was 1 mgm.; each succeeding dose was doubled unless the condition of the horse contra-indicated it. After each three day period, the horse was allowed to rest for seven day. The first three doses of the bacillus emulsion were heated at 55°C. for one hour before inoculation. Later, living cultures were inoculated. From four to six series of inoculations were given each horse.

In table 1 are given only the strains which were used by us to test the protective action of our monovalent serum.

[3] Reported in Arch. Ped., 1921, **38**, 329.

With the serums obtained, we corroborated fully the work of Durand showing that there are at least five agglutinative types of diphtheria bacilli.

Havens kindly sent us four strains of his group II and we found that none of them agglutinated with our "no. 8" serum.

Havens did not mention Durand's work in his article. He reported the results of his study of 206 strains. These were from acute cases, convalescents and carriers. All varieties of morphologic cultures were tested but he says that "morphology bears no observed relation to agglutinating properties." A certain strain was chosen to inoculate rabbits for the production of agglutinating serum. Havens does not give the dosage or the course. He simply states that the injections were made intravenously in increasing amounts, and that an agglutinating serum was obtained with a titer of 4860 for the homologous strain. Of the series of 206 strains, 169 agglutinated in this serum to the same titer as the homologous strain, while 37 failed to agglutinate in any dilution. One strain of this second group was chosen to inoculate a rabbit for the production of agglutinating serum. This serum gave a titer of 4800 or higher. The other 36 strains of this group agglutinated to the same titer. He concludes as follows:

> Judging by the evidence furnished by the agglutination test there are two biologic groups of the diphtheria bacillus. No evidence of cross agglutination was found among the members of this series [obtained in Iowa City].
>
> All members of the series fell into one of the two groups with no differences observed in the degree of agglutinability. As judged by the results of agglutination, two groups exist which include all strains of the diphtheria bacillus.

Havens said that the virulence in animals was approximately the same in the two groups.

When we consider the results of Havens in connection with Durand's and those of our own, it seems fair to infer that in the comparatively small city from which he drew his material, only two types of bacilli were present.

There can be no doubt whatsoever that in all large communities there are many types and at least several dominant ones.

II. PROTECTIVE ACTION OF DIPHTHERIA ANTITOXIN (STANDARD NO. 8), AGAINST DIFFERENT AGGLUTINATING TYPES OF B. DIPHTHERIAE[4]

The question of the protection results is so important that we quote Havens' experiments in detail. In attempting to show "the protective properties of diphtheria antitoxin with reference to biologic groups," he reports first the following tests with various cultures and diphtheria antitoxin no. 8.

Havens' tests

CULTURE	GROUP	AMOUNT	UNITS ANTITOXIN	TYPE ANTITOXIN	RESULT
		cc.			
210	I	1	None		Died, 48 hours
210	I	1	100	I	Lived
221	II	1	None		Died, 36 hours
221	II	1	100	I	Died, 36 hours
141	II	1	None		Died, 48 hours
141	II	1	100	I	Died, 48 hours

The results of these tests of Havens indicate that group I antitoxin has no neutralizing effect upon the toxins produced in the animals by group II cultures. Similar results followed when he injected cultures intracutaneously. Six strains of group I bacilli were inoculated into guinea pigs that had been injected with 100 units of group I antitoxin. No cutaneous lesions were produced. Eighteen strains of group II bacilli were inoculated into guinea pigs that had received 100 units of group I antitoxin. The reactions in 15 were identical with the controls, while those in three were slightly less than the controls.

Before discussing Havens' results with toxin, we give in table 2 our findings with cultures of at least five different agglutinating types including three of Havens' "group II" strains. The cultures were heavy eighteen hour growths in veal broth.

[4] We were assisted in this part of the work by Jane L. Berry, Harriet L. Wilcox and Charles Greenwald.

TABLE 2

Life-saving effects of type I antitoxin in animals injected with bacilli of various types

TYPE	WEIGHT OF GUINEA PIG	AMOUNT OF CULTURE	UNITS OF ANTITOXIN NO. 8	RESULT
	grams	*cc.*		
Type I. (Our no. 8, New York City)	248	2.0	None	Died 3 days
	230	3.0	None	Died 2 days
	355	3.0	None	Died 2 days
	230	3.0	0.2	Died 2 days
	230	3.0	0.5	Died 2 days
	360	3.0	1.0	Remained well
	355	3.0	2.0	Remained well
Type III. (Durand's Nodet, France)	225	0.2	None	Died 2 days.
	225	0.2	0.5	Remained well
Type V. (Durand's Sir-beaux, France)	235	0.2	None	Died 9 days
	400	0.75	None	Died 4 days
	235	0.2	0.2	Died 8 days
	405	0.75	1.5	Remained well
Type IV. (No. 11, New York City)	242	2.0	None	Died 3 days
	245	2.0	0.5	Remained well
Type IV. (Durand's Benjamin, France)	240	0.05	None	Died 2 days
	235	0.1	0.2	Died 6 days
	235	0.05	0.05	Remained well
No. 3362, unclassified (New York City)	245	0.2	None	Died 3 days
	255	0.2	0.2	Died 4 days
	255	0.2	0.5	Remained well
B, unclassified (Near New York City)	235	0.1	None	Died 6 days
	245	0.1	0.2	Remained well
No. 151 (Havens' group II, Iowa City)	237	1.0	None	Died 3 days
	262	1.0	None	Died 2 days
	255	1.0	0.2	Remained well
	245	1.0	0.5	Remained well
No. 184 (Havens' group II, Iowa City)	225	0.2	None	Died 2 days
	232	0.2	None	Died 2 days
	245	0.2	0.2	Died 6 days
	230	0.2	0.5	Remained well
No. 8347 (Havens' group II, Iowa City)	217	0.1	None	Died 10 days
	440	0.3	None	Died 2 days
	235	0.3	0.5	Died 3 days
	225	0.3	1.0	Died 3 days
	215	0.3	5.0	Remained well
	255	0.3	5.0	Remained well

The results given in the table do not show any striking difference in the protecting value of the type I antitoxin, when used in animals inoculated with a type I strain or with strains of other types. All who have worked extensively with living cultures have discovered that the results are much more irregular than when toxins are used. The cultures vary from day to day, according to the culture medium used, in the abundance of their growth and in the vigor and apparent toxicity of the bacilli; a much larger number of units is required to protect against a fatal dose of living bacilli than against a fatal dose of toxin. There is nothing in our results to indicate to us that there is any marked difference in the pathogenic power of the different types of bacilli.

The last three cultures are of unusual interest since they were sent us by Havens as examples of his Group II strains. All produced toxin of moderate strength when grown in bouillon. It is to be noted that our antitoxin produced by type I no. 8 bacillus was capable of protecting the animal which had received a subcutaneous injection of an amount of culture which produced death in the control animal within two days. Indeed, the type I antitoxin appears to be as efficient against two of the cultures sent by Havens as against strain "no. 8." Although with the third culture a considerably larger quantity of antitoxin was required, it is noted below in table 3 that the toxin produced by this culture was neutralized by the usual amount of antitoxin.

The experiments of Havens showing the neutralizing results of mixtures of his group I antitoxin and group II toxin are given in the following tabulation:

Havens' tests

STRAIN NUMBER	TOXIN GROUP	MINIMAL LETHAL DOSES	GROUP I ANTITOXIN UNITS	RESULT
53	II	2	10	Died, 72 hours
53	II	2	—	Died, 72 hours
74	II	2	5	Died, 48 hours
74	II	2	20	Lived, marked virulence
74	II	2	25	Lived, oedema
74	II	2	50	Lived, no lesions
34	I	2	5	Remained well
34	I	2		Died in 48 hours

The group II toxin required far larger amounts of group I antitoxin to neutralize it than did the group I toxin.

Neutralizing experiments with mixtures of group II antitoxin and group I toxin.

STRAIN NUMBER	TOXIN GROUP	MINIMAL LETHAL DOSE	GROUP II ANTITOXIN UNITS	RESULT
34	I	1	5	Died, 96 hours
34	I	1		Died, 96 hours

As seen in the above tabulation, one minimal lethal dose of group I toxin was not neutralized by five units of group II antitoxin.

The fact that in Havens' tests very large amounts of group I antitoxin usually neutralized 2 minimal lethal doses of group II toxin, indicates that the group I antitoxin contained the same

TABLE 3

Toxin results

TOXIN		WEIGHT OF GUINEA PIG	NUMBER OF MINIMAL LETHAL DOSES	NO. 8 ANTITOXIN	RESULT
From culture	Type				
		grams			
No. 8	I	250	1.0	0.02	Lived
Durand	II	230–260	1.0	0.02	Lived
Nodet	III	230–260	1.5	0.02	Lived
Benjamin	IV	230–260	2.0	0.02	Lived
No. 11	IV	230–260	1.0	0.02	Lived
3362	Unclassified	230–260	2.0	0.02	Lived
151	Havens, group II	230–260	1.0	0.02	Lived
8347	Havens, group II	230–260	1.5	0.1	Lived
8347	Havens, group II	230–260	100.0	10.00	Lived

neutralizing substances for group II toxin as for group I toxin but that there were quantitative differences in the proportion of the different antitoxic substances.

Havens stated "the results may throw some light on those cases of diphtheria which are not benefited except by large amounts of antitoxin." He alluded also to the death rate re-

maining at 10 per cent in cases of diphtheria. He suggested adding his group II toxin to horse injections, to the Schick test toxin and also to toxin-antitoxin mixtures.

Table 3 gives the results which we obtained with neutralizing mixtures of our own antitoxin no. 8 and the types of toxin indicated.

It is evident from this table that with the strains obtained from New York, from France and from Iowa no such difference has been discovered, as Havens believes he found. It would take repeated experiments and many animals to prove whether or not there is any minor difference either quantitatively or qualitatively between these toxins produced by the different agglutinative types.

CONCLUSIONS

The group of diphtheria bacilli contain strains belonging to several agglutinative types.

The toxins formed by these different types are, however, qualitatively alike and, from the practical standpoint, quantitatively so. Whether or not there are slight quantitative differences, further studies will be necessary to show.

Strong toxin from any diphtheria bacillus strain is suitable for the Schick test and for immunization of man or animal.

A monovalent antitoxic serum is suitable for protective and curative measures against all diphtheria strains.

REFERENCES

(1) Havens, L. C.: Jour. Inf. Dis., 1920, **26**, 388.
(2) Schwoner, J.: Wien. Klin. Woch., 1902, **15**, 1274.
(3) Langer, H. Centralbl. f. Bakt., 1916, Orig., **78**, 117.
(4) Mason, E. H.: Mil. Surgeon, 1919, **45**, 560.
(5) Durand, Paul: Compt. Rendus Soc. Biol., 1918, **81**, 1011 and 1920, **83**, 611 and 613.

THE RELATIONSHIP OF LIPOIDS AND PROTEINS TO SERUM REACTIONS IN TUBERCULOSIS

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While working with sera of tuberculous patients[1] it became necessary to note the effect of (*a*) the removal of lipoids and (*b*) the precipitation of globulins on certain serum reactions. The results obtained seemed sufficiently clear cut for publication in the present paper.

PART A. LIPOIDS IN TUBERCULO SERUM REACTIONS

The relationship of lipoids to immunity reactions has been a subject of great interest in recent years. Krumwiede and Noble (1) have recently been unable to confirm the claim made by Stuber that agglutinins are lipoidal in nature. Levaditi and Yamanouchi (2) have claimed that the substances in syphilitic sera responsible for the Wassermann reaction, can be extracted with alcohol. Noguchi (3) was unable to confirm this observation; he concluded that the reacting substance in syphilitic serum was non-lipoidal in character. Kolmer and Pearce (4) found that ether and chloroform narcosis tended to diminish the concentration of non-specific fixation bodies in normal, inactivated dog and rabbit serum. Observations on the serum of humans, withdrawn during and immediately following ether narcosis, show that there may be a profound alteration of the

[1] This work was carried out through the coöperation of facilities at the Research Division of the Connaught Antitoxin Laboratories, the Department of Soldiers' Civil Reëstablishment Chest Clinic, and the Department of Medicine, University of Toronto. It forms part of the investigation of clinical and laboratory data in tuberculosis which Doctor Caulfeild and his associates are conducting, the results and conclusions of which will be published later.

Wassermann reaction under these conditions. It seems likely, then, that alterations in the lipoidal content of serum must have an important bearing on this reaction. Kolmer (5) further showed that extraction of normal rabbit and dog serum with ether tends to remove a large portion of the serum constituents responsible for non-specific fixation with lipoidal and bacterial antigens; he also found that feeding lecithin to these animals was followed by increased power of the inactivated serum to adsorb complement. Noguchi (6) has shown that only sera containing lecithin, fatty acids or soaps are capable of activating cobra venom (rendering it hemolytic). Venom hemolysis indicates the possible important relationship of lipoids to hemolytic complement, venom containing the hemolytic amboceptor and the complement probably being derived from corpuscular lecithin (Kolmer). Calmette, Massol and Breton (7), studying the activating power of different sera on cobra venom, found that inactivated sera of tuberculous patients very frequently showed this characteristic. Calmette (8) attempted to use this reaction in the diagnosis of tuberculosis. In 77 sera from tuberculous patients the results obtained were as follows: turban 1, reaction + in 76 per cent; turban 2, reaction + in 57 per cent; turban 3, reaction + in 70 per cent. In 26 normal sera: reaction + in 37 per cent. He also observed this reaction in the serum of patients suffering from syphilis, in cerebrospinal meningitis, in Addison's disease, in general paralysis of the insane, and in severe maladies associated with more or less profound affection of the nerve cells or suprarenal capsules. Free lecithin was also found to occur in the serum of anaesthetized subjects. Finally the inactivated serum of certain animals is normally active; the horse, dog, rat, goat and rabbit are in this class. Kolmer (5) has drawn attention to the frequency with which non-specific complement fixation bodies are present in normal inactivated dog and rabbit serum and he has shown that extraction with ether tends to remove these bodies. Calmette's observations show that the serum of these animals normally contains large amounts of lecithin, fatty acids or soaps; this goes to confirm

Kolmer's opinion that the substances responsible for the non-specific fixation obtained with these sera are lipoidal in character.

It is evident from even such an incomplete review of the literature that lipoids have a not inconsiderable influence on the Wassermann reaction (although the weight of opinion considers the reacting substances non-lipoidal in nature), that the lipoidal content of certain animal sera favors conditions for the development of non-specific fixation and finally that the lipoidal content of most tuberculous sera is higher than normal. Our own work has been an attempt to show in what way the lipoidal content of tuberculous sera is related to the complement fixation reaction in tuberculosis and to Caulfeild's (9) inhibitive reaction.

Methods

The object of this part of the work was to extract the lipoids from the serum as completely as possible and to note the effect of this removal on the reactions under examination. In the earlier work an attempt was made to extract the lipoids by thoroughly mixing the serum with petroleum ether (4 volumes of petroleum ether to 1 volume of serum). After extraction the serum was separated by centrifugalization. The results with this method, however, proved quite indeterminate in a series of 90 sera tested. It was thought that the method employed might remove the lipoids only very incompletely, accordingly Friedemann and Herzfeld's method (10), slightly modified, was used. Five-tenths cubic centimeters of serum were spread on a filter paper and dried in an electric oven at 45°C. for one hour. 1 volume dried in this way was reserved for a control; to the other volume in a test tube was added a mixture of equal parts of absolute alcohol, ether and chloroform. The tubes were shaken half an hour after which the mixture was poured off and the filter paper dried at 37°C. Saline was then added to the dried filter paper to make a dilution 1:5; the filter paper was worked into a pulp, the liquid pressed out, poured into another tube and centrifugalized to remove the particles of filter paper. The control portion of the serum was treated in the same way except that the extraction with alcohol, chloroform and ether was omitted.

1. Effect of extraction of lipoids on the complement fixation reaction

Table 1 contains the results obtained, when fifteen sera from tuberculous patients, showing varying degrees of fixation,[2] were were extracted in this way.

Comment. The results in the control untreated fraction (dilution 1:10) and in the control dried fraction (dilution 1:5) were quite comparable, there being only a moderate loss of fixation bodies in the latter. The dried and extracted sera, however, in

TABLE 1

UNTREATED SERUM, 1:10	SERUM DRIED AND REDISSOLVED IN SALINE, 1:5	SERUM DRIED, EXTRACTED AND REDISSOLVED IN SALINE, 1:5
*3-1-0-0	2-1-0-±	C. H.
3-±-0-0	3-2-±-1	1-0-0-±
4-2-±-0	4-3-2-2	2-1-0-1
4-3-2-0	4-3-1-2	1-0-0-1
4-3-±-0	4-3-0-0	C. H.
4-3-1-0	4-4-3-1	C. H.
4-2-0-0	4-4-2-2	C. H.
4-2-±-0	4-3-1-1	C. H.
4-4-3-0	4-4-2-0	C. H.
4-4-3-0	4-4-3-±	C. H.
4-4-3-0	4-4-3-0	C. H.
4-4-4-0	4-4-4-2	C. H.
4-3-2-0	4-4-4-0	2-1-0-1
3-±-0-0	3-2-±-0	2-±-0-2
4-2-0-0	4-1-0-0	C. H.

* 4 = no hemolysis; 3 = 25 per cent hemolysis; 2 = 50 per cent hemolysis; 1 = 75 per cent hemolysis; ± = almost complete hemolysis; 0 and C. H. = complete hemolysis. First three tubes contain 0.1 cc. of serum and antigen, and two, two and a half and three units of complement. The fourth tube is a control containing 0.2 cc. of serum and 2 units of complement.

almost every instance, showed a marked loss of power of fixation. In many instances the loss of power of fixation was complete. It is evident then, that extraction in this manner either destroys or removes[3] substances wholly or partially responsible for the

[2] Petroff's whole bacillus antigen was used throughout for the complement fixation tests.

[3] An attempt was made to test the alcohol, chloroform, ether extractives for complement fixation bodies without success on account of the high anti-complementary power of this fraction.

development of the complement fixation reaction in sera of tuberculous patients.

It was thought that comparable results might be obtained by extraction of sera of animals immunized with the tubercle bacillus. The results of extraction of such fixation guinea-pig and rabbit sera, however, failed to demonstrate a definite loss of power of fixation in these instances. Assuming that the fixation bodies are of similar chemical constitution in human, guinea-pig and rabbit sera, these results are puzzling. The fact that the sera of the artificially immunized animals showed a higher concentration of fixation bodies than the human tuberculous sera may be a possible explanation, as here the extraction of lipoids may be incomplete. It is true that the method employed does not completely free sera of lipoids. This has been demonstrated by Suranyi (11) who followed the technic of Friedemann and Herzfeld closely and then extracted the supposedly lipoid-free serum in a soxhlet apparatus. He found that frequently as much as 25 per cent of the serum lipoids remained after extraction by the method of Friedemann and Herzfeld.

2. Effect of the extraction of lipoids on the inhibitive[4] reaction

Thirty inhibitive sera were extracted by the method described. There was no evidence to show that extraction removed the active substances. It was found however, that there was a quite marked loss of inhibitive power in the sera that had been dried and redissolved without extraction. The extracted sera showed a similar, but not greater, loss of inhibitive power. It is evident

[4] The technic of this reaction has been fully described by Caulfeild (9). Briefly, it is found that certain human sera, combined with anti-complimentary doses of antigen (AE extract of the tubercle bacillus) and two and a half units of complement, will free the complement from its non-specific adsorption by the antigen. In our tests four tubes are used, the first three tubes containing varying doses of antigen, two and a half units of complement and 0.1 cc. of inactivated serum from which the natural amboceptor has been previously removed by extraction with sheep cells, the fourth tube is the ordinary serum anti-complementary control. A very strong inhibitive serum will show hemolysis in all tubes. A strong inhibitive serum will give a 4-0-0 reading; a weak inhibitive serum will give a 4-4-0 reading and a serum containing no inhibitive substance will give a 4-4-4 reading.

then that the components of the dried sera were only very incompletely redissolved in saline, the inhibitive fraction remaining largely insoluble on the filter paper. Caulfeild (9) observed a similar loss of inhibitive power when antigen-serum mixtures were dried on filter paper and redissolved.

The researches of Jobling and Petersen (12) in connection with the increased antitryptic power of guinea-pig serum following anaphylactic shock, suggested an indirect method of determining the effect of lipoids on the inhibitive reaction. These authors believe that the increased antitryptic power of guinea-pig serum immediately following anaphylactic shock, especially if protracted, is due to an increased concentration of the unsaturated lipoids in the serum. The increased antitryptic power is explained by the liberation of lipoids following cellular destruction. Rusjnjak (13) has shown that the antitryptic power of serum following anaphylactic shock is frequently raised as much as 100 per cent; the optimal conditions for the development of this increase are obtained when an incubation period of from fifteen to thirty minutes intervenes before shock symptoms appear.

3. Effect of anaphylactic shock on the inhibitive reaction

In the following experiments anaphylactic shock of varying severity was induced in guinea-pigs and the inhibitive power of the serum[5] before and after shock was determined.

It is not possible to give the protocols of these experiments in detail. The most important single fact is the length of the latent period before the onset of shock symptoms. In table 2 the results are shown of the study of these sera before and after anaphylactic shock; the sera from animals showing a latent period of fifteen minutes or more before the onset of shock symptoms are grouped together. It is here that one would expect to find the inhibitive power increased if it is due to unsaturated serum lipoids.

[5] Guinea-pig serum was ideal to work with here because normal guinea pig serum often gives a weak inhibitive reaction, hence we know that conditions are favorable for the development of the reaction and for the perception of varying amounts of inhibitive substance.

Comment. Sera 1 and 4 show an increased inhibitive power following anaphylactic shock, but this occurs nowhere else in the series. Sera 9 to 13 should develop ideal conditions for increased antitryptic power in the serum as the latent period was fifteen minutes or over in each of these animals; yet these sera show no definite increase in inhibitive power. In this series of

TABLE 2

NUMBER	INHIBITIVE REACTION BEFORE SHOCK	INHIBITIVE REACTION FOLLOWING SHOCK	REMARKS
Latent period less than fifteen minutes			
2	4-4-4-0	4-4-4-0	Moderate shock
3	4-4-4-0	4-4-4-0	Moderate shock
5	4-4-4-0	4-4-4-0	Sudden, fatal shock
6	4-4-4-0	4-4-4-0	Mild shock
7	4-4-4-0	4-4-4-0	Moderate shock
8	4-4-4-0	4-4-4-0	Severe shock
Latent period fifteen minutes or more			
1	4-4-4-0	4-2-0-0	Very mild shock
4	4-4-4-0	4-4-0-0	Mild shock
9	4-4-4-0	4-4-4-0	Mild shock
10	4-4-4-0	4-4-3-0	Mild shock
11	4-4-4-0	4-4-3-0	Mild shock
12	4-4-4-0	4-4-4-0	Mild shock
13	4-4-4-0	4-4-4-0	Mild shock
14	4-4-4-0	4-4-4-0	Moderate shock
15	4-4-4-0	4-4-4-0	Moderate shock
16	4-4-4-0	4-4-4-0	Moderate shock
17	4-4-4-0	4-4-4-0	Moderate shock
18	4-4-4-0	4-4-4-0	Moderate shock
19	4-4-4-0	4-4-4-0	Moderate shock

animals anaphylactic shock of all degrees was encountered, from the severe and immediately fatal type to the almost imperceptible type. The animals which showed the increased inhibitive power were only very mildly shocked. As this increase occurred definitely in only two cases in twenty it seems very unlikely that it can be associated with the increased lipoidal content which is supposed to occur so frequently under these conditions.

PART B. PROTEINS IN TUBERCULO SERUM REACTIONS

The rôle of proteins in certain immunity reactions is well recognized, in others less definitely so. Huntoon (14) and others, in an extensive study of the chemical nature of agglutinins, bactericidal and protective substances in immune sera, concluded that such antibodies were colloidal in nature; they were not affected by trypsin over considerable periods, were not in the euglobulin or pseudoglobulin fractions and were not soluble in ether.

The relationship of proteins to complement fixation has not been definitely determined. Noguchi (3) found that sera of untreated syphilitics almost universally showed an increased globulin content; there was, however, no complete parallelism between this increased globulin content and the positive Wassermann reaction. Rowe (15) found an increased globulin content in sera in all infections with the exception of acute tonsillitis, typhoid fever and chronic bronchitis. He also found that the Wassermann reaction is not dependent on a quantitative increase of serum globulins. Kapsenberg (16) has recently shown that the fixation bodies responsible for the development of the Wassermann reaction are completely removed when the globulin fraction is precipitated with ammonium sulphate and that they can be demonstrated in the globulin fraction. Nishida and Petroff (17) in a study of fixation bodies in tuberculous sera concluded that these substances are either globulins or that they are adsorbed by the globulin fraction of the serum when it is precipitated by ammonium sulphate. Calmette (18) working with the serum of cows immunized with the tubercle bacillus, precipitated the euglobulin fraction by bubbling carbon dioxide through the diluted serum and found that the fixation bodies remained in the supernatant fluid.

In the present work euglobulin was precipitated by bubbling carbon dioxide through serum[6] diluted 1:10 with distilled water till a heavy turbidity developed. The sera were allowed to stand

[6] Sera were inactivated at 56°C. for half an hour and when the inhibitive reaction was being investigated all sera were previously extracted with sheep cells to remove the natural amboceptor.

for two hours till a flocculent precipitate was formed; they were then centrifugalized. The supernatant fluid was poured off and rendered isotonic. The precipitate was redissolved in saline to give the original 1:10 dilution.

1. Effect of precipitation of euglobulin on the complement fixation reaction in tuberculosis

The results of the precipitation of euglobulin in human sera showing various degrees of fixation and in the sera from a rabbit and a guinea pig which had previously been immunized with the tubercle bacillus, are shown in table 3.

TABLE 3

SERUM	UNTREATED SERUM	TREATED SERUM	
		Euglobulin fraction	Supernatant fluid
Complement units	Control tube 2 2-3-4-5-6-7	Control tube 2 2-3-4-5-6-7	Control tube 2 2-3-4-5-6-7
Human no. 19	4-3-2-0-0-0-0	3-0-0-0-0-0-0	4-4-3-0-0-0-0
Human no. 13	4-4-4-4-4-4-0	4-2-0-0-0-0-0	4-4-4-4-4-4-0
Immunized rabbit	4-4-4-4-4-4-0	2-0-0-0-0-0-0	4-4-4-4-4-4-0
Immunized guinea-pig	4-4-4-4-4-4-0	4-3-0-0-0-0-0	4-4-4-4-4-4-0

Comment. The results are definite and uniform. The fixation bodies in the supernatant fluid are in practically the same concentration as in the untreated serum. There is an occasional and slight amount of fixation in the euglobulin fraction. As the control tubes of the supernatant fluid hemolyzed much more rapidly than those of the euglobulin fraction, the slight and irregular fixation in this fraction is likely due to the antilytic power of the euglobulin plus the antilytic power of the antigen.

Renaux (19) separated the euglobulin from syphilitic sera using this method. He found that the fixation bodies remained in the supernatant fluid and appeared only irregularly in the euglobulin precipitate. Renaux in addition made a very interesting observation. He separated the diluted serum into two portions to one of which he added a small amount of lipoid emulsion (cholesterinized heart extract); the portions of diluted serum

were now treated with carbon dioxide in the ordinary way. Tests with the supernatant fluid and euglobulin for fixation bodies now showed that in the case of the serum previously treated with lipoids the fixation bodies had been completely transferred to the euglobulin sediment; in the serum free from lipoids the fixation bodies of course remained in the supernatant fluid. We were able to repeat Renaux's experiment with the same result; a sample protocol is shown in table 4.

Comment. It is seen, that, by the addition of lipoidal emulsion (Wassermann antigen) to the serum before the precipitation of euglobulin, the fixation bodies are transferred to the euglobulin sediment. There seem to be two possible explanations of this phenomenon: (*a*) The lipoids added are either adsorbed by the

TABLE 4

	EUGLOBULIN	SUPERNATANT FLUID
Without lipoid emulsion	*0-0-±-1-2-0	0-2-4-4-4-0
Lipoid emulsion added before precipitation of euglobulin	0-2-4-4-4-4	0-0-0-0-0-0

* The first five tubes contain antigen, 3 units of complement and varying amounts of 1,10 serum; i.e., 0.05, 0.1, 0.3, 0.5 and 1.0 cc. The sixth tube is a control containing 2.0 cc. of serum and 3 units of complement.

euglobulin sediment or actually combine with it to form a lipoprotein, or (*b*) The increased concentration of lipoids following the addition of antigen to the diluted serum may result in the precipitation of the fixation bodies with the euglobulin fraction. If explanation (*a*) is correct, then the lipoids in a state of emulsion are simply adsorbed by the flocculating euglobulin and the fixation body is carried down in combination with its antigen. If explanation (*b*) is correct, then the fixation body is primarily precipitated and the antigen is pulled down in combination with the fixation body. Assuming explanation (*b*) to be possible it is at once apparent that a similar phenomenon might be obtained by the addition of lipoidal emulsion to sera containing tuberculo-complement fixation bodies. Accordingly human, rabbit and guinea-pig fixation sera were tried out in this way. It was found that the addition of lipoidal emulsion (Wassermann anti-

gen and AE extract[7] of the tubercle bacillus) to these sera, before treatment with carbon dioxide, has practically no effect on the supernatant fluid. Either, then, we are dealing with a different type of fixation body in tuberculous sera which is not precipitated, or the fixation bodies in syphilitic sera simply combine with the antigen immediately and are precipitated after the combination has occurred.

2. Effect of precipitation of euglobulin on the inhibitive reaction

A further explanation of the inhibitive reaction seems essential at this point. Caulfeild described his inhibitive reaction as present in human sera in various degrees, but a strong inhibitive reaction (4-0-0 or stronger) was considered pathological and in cases of tuberculosis was associated with a favourable prognosis. Simultaneously Calmette described an *inhibiteur* reaction in the serum of animals hyperimmunized to the tubercle bacillus. The method of demonstration of Calmette's reaction (20) differs from the method described by Caulfeild. It will be recalled that Caulfeild used as his antigen an alcohol-ether extract of the tubercle bacillus (AE extract). In this reaction four tubes are used for each serum, the first three containing varying doses of antigen and the fourth being the customary serum control for anti-complementary power. Of the tubes containing antigen, the third contains the smallest dose which alone just completely adsorbs two and one-half units of complement, the second contains an amount which adsorbs five units of complement and the first contains sufficient antigen to alone adsorb ten units of complement. The first three tubes each receive 0.1 cc. of serum (previously treated with sheep cells to remove natural amboceptor) and the fourth 0.2 cc. of serum. Each tube receives two and one-half units of complement. The periods of incubation are the same as those used for the tuberculo-complement fixation test. When 0.1 cc. of human serum reverses the antilytic power

[7] Fixation with this antigen is only very rarely obtained with sera from tuberculous sources. The sera employed in the above experiment all gave negative fixation reactions with this antigen; thus the factor of the combination of the antigen and fixation body is eliminated in this experiment.

of the smallest dose of antigen so that complete hemolysis results in the third tube (4-4-0) this is considered a normal inhibitive reaction. When, however, the inhibitive power is strong enough to produce complete hemolysis in the second and third tubes (4-0-0) or partial hemolysis in the first tube (2-0-0), this is considered a pathological reaction. Calmette used as his antigen an aqueous extract of the tubercle bacillus (B_1); as this antigen is not anticomplementary in as low a dilution as 1:5 it is at once apparent that it could not be used by Caulfeild's method. Calmette demonstrated his reaction by combining sera of hyper-immune animals with his B_1 antigen. The *inhibiteur* serum and antigen were given a preliminary incubation of half an hour and then complement and the fixation serum were added and the complement fixation test proceeded with as usual. He found that in such a combination the fixation was completely masked.[8]

We have recently been able to demonstrate a similar reaction[9] in human sera. Many sera of tuberculous humans which give a strong positive fixation with Petroff's antigen will also give a positive or strong positive fixation with Calmette's B_1 antigen. When these sera are combined with certain human sera the fixation is found to be inhibited. Sera giving a strong inhibitive reaction with Caulfeild's method almost always give a strong *inhibiteur* reaction with Calmette's method also. The evidence thus far, then, points to the identity of these two reactions. Calmette separated the euglobulin from his hyper-immune sera and found that the inhibitive substance was contained in this fraction. We precipitated euglobulin from human sera, from the serum of normal rabbits and guinea-pigs and from the serum of rabbits and guinea pigs immunized with the tubercle bacillus. The results are shown in table 5.

Comment. It is evident from this table that the inhibitive substance in sera is almost completely precipitated in or with the

[8] Caulfeild (21) in his earlier work encountered an occasional serum which gave a positive fixation with an alcohol ether extract of the tubercle bacillus. Combinations of such sera with inhibitive sera in the presence of AE extract antigen showed the inhibitive sera were, when strong enough, able to mask the fixation.

[9] These results will be published in a separate paper.

euglobulin fraction. This is true not only for the inhibitive substance present in pathological sera but also for that present in normal human and normal guinea-pig serum. The pathological inhibitive reaction is evidently due, then, to an increased concentration of a substance normally present in human serum in small amounts. The effect of the precipitation of euglobulin is

TABLE 5

SERUM	UNTREATED SERUM	TREATED SERUM	
		Euglobulin fraction	Supernatant fluid
Normal human	4-4-0-0	4-4-0-0	4-4-0-0
	4-4-0-0	4-4-0-0	4-4-4-0
	4-3-0-0	4-3-0-0	4-4-4-0
Pathological human (tuberculosis)	4-2-0-0	4-0-0-0	4-4-2-0
	4-2-0-0	3-0-0-0	4-4-3-0
	4-2-0-0	4-0-0-0	4-4-0-0
	4-0-0-0	2-0-0-0	4-4-4-0
	4-1-0-0	1-0-0-0	4-4-0-0
	4-1-0-0	4-0-0-0	4-4-1-0
Normal rabbit	4-4-4-0	4-4-4-0	4-4-4-0
	4-4-4-0	4-4-4-0	4-4-4-0
	4-4-4-0	4-4-4-0	4-4-4-0
	4-4-4-0	4-4-4-0	4-4-4-0
	4-4-4-0	4-4-4-0	4-4-4-0
Immunized rabbit	4-4-4-0	4-3-0-0	4-4-4-0
	4-4-4-0	4-4-0-0	4-4-4-0
	4-4-4-0	4-4-2-0	4-4-4-0
	4-4-4-0	4-3-0-0	4-4-4-0
	4-4-4-0	4-4-0-0	4-4-4-0
	4-4-4-0	4-3-0-0	4-4-4-0
Normal guinea pig 1	4-4-1-0	4-4-0-0	4-4-4-0
Normal guinea pig 3	4-4-2-0	4-4-0-0	4-4-4-0
Normal guinea pig 5	4-4-1-0	4-4-0-0	4-4-4-0
Normal guinea pig 6	4-4-3-0	4-4-0-0	4-4-4-0
Immunized guinea pig 1	4-4-3-0	4-0-0-0	4-4-4-0
Immunized guinea pig 3	4-3-2-0	4-0-0-0	4-4-4-0
Immunized guinea pig 5	4-4-3-0	4-4-0-0	4-4-4-0
Immunized guinea pig 6	4-3-1-0	±-0-0-0	4-3-0-0

identical on Caulfeild's inhibitive substance and on Calmette's *inhibiteur* substance. Calmette finds that the euglobulin fraction of his hyperimmune sera contains a substance which, when combined with a positive fixation serum, masks the complement binding power of this serum in the presence of B_1 antigen. The results in table 5 show that the euglobulin fraction of certain human sera and of the serum of immunized animals contains a substance which reverses the antilytic power of an alcohol ether extract of the tubercle bacillus and allows hemolysis to proceed where ordinarily no hemolysis would occur. It is of interest to note that the inhibitive power of the serum of the immunized animals is increased over normal although this is not always revealed till the euglobulin fraction is precipitated.

PART C

Discussion

The evidence presented indicates that the fixation bodies in human tuberculous sera are lipoidal in character because:

1. Extraction of dried sera with alcohol, chloroform and ether removes these bodies almost completely.

2. When the sera are dried on filter paper and redissolved in saline there is only a moderate loss of fixation power. This indicates that the active substances redissolve quite readily; protein does not redissolve readily after drying.

3. Fixation bodies occur only irregularly in the euglobulin fraction of the serum; they occur in the supernatant fluid in the same concentration as in the untreated serum.

At first sight these observations are opposed to those of Nishida and Petroff who found that the tuberculo-complement fixation bodies appeared in the globulin sediment precipitated by ammonium sulphate. Kapsenberg also found that the fixation bodies responsible for the Wassermann reaction were precipitated in this way. Our own observations have shown, however, that the complete precipitation of fixation bodies in syphilitic sera is not necessarily accompanied by the complete precipitation of globulin. This is shown by the fact that almost complete precipi-

tation of syphilitic fixation bodies is obtained when lipoidal emulsion (Wassermann antigen) is added to the diluted serum before the precipitation of euglobulin by carbon dioxide. In this experiment only the euglobulin is precipitated, the sediment is not increased in amount, and the remaining globulin fraction can be demonstrated in the supernatant fluid by half saturation with ammonium sulphate. It is evident, then, that the fixation bodies in syphilitic sera are not globulins but are merely carried down with the globulin fraction when it is completely precipitated. It seems altogether likely that the precipitation of the tuberculo complement fixation bodies in the globulin sediment obtained with ammonium sulphate is of a similar nature.

The inhibitive bodies in human and certain animal sera appear to be protein in character because:

1. They are almost completely precipitated in the euglobulin fraction of the serum.

2. The inhibitive power of guinea-pig serum is not regularly increased following protracted anaphylactic shock when the unsaturated lipoids of the serum are increased. The two sera which showed an increased inhibitive power in our experiments quite possibly contained an increased euglobulin content. It is possible that cellular disruption not only increased the unsaturated lipoids (from the cell wall) in the serum but also occasionally the euglobulin.

3. They are not removed by alcohol, chloroform and ether extraction of dried serum; serum, however, which is dried and redissolved in saline shows a marked loss of inhibitive power. This indicates that the substances responsible for the inhibitive reaction are protein in character as the proteins of dried serum redissolve again only incompletely.

The identity of the substances responsible for the inhibitive reaction of Calmette and that of Caulfeild seems certain. It is true that there are minor differences but we have found that the active substance in Caulfeild's reaction is precipitated in the euglobulin fraction; Calmette finds that the *inhibiteur* substance in his hyperimmune sera is precipitated in the euglobulin fraction also. We have further found that human sera exhibiting a

strong inhibitive reaction by Caulfeild's method also give a strong *inhibiteur* by Calmette's method. The fact that the substance responsible for Caulfeild's reaction is increased when animals are immunized with the tubercle bacillus, further identifies it with the substance responsible for Calmette's reaction. Calmette finds *inhibiteur* in high concentration in the serum of hyperimmunized cows. We have found (table 5) that there is a moderate increase of Caulfeild's inhibitive substance in the serum of immunized rabbits and a more marked increase in the serum of immunized guinea pigs.

Conclusions

1. Evidence is introduced which indicates that the fixation bodies in human tuberculous sera are either removed or destroyed by extraction of the dried serum with alcohol, chloroform or ether.

2. The substances responsible for the inhibitive reaction of Caulfeild are contained almost wholly in the euglobulin fraction of the serum.

3. Evidence is introduced to show that the substances responsible for the *inhibiteur* reaction of Calmette are identical with those responsible for the inhibitive reaction of Caulfeild.

REFERENCES

(1) Krumwiede, C. and Noble, C.: Jour. Immun., 1921, **6**, 201.
(2) Levaditi and Yamanouchi, T.: Compt. Rend. Soc. de Biol., 1908, **64**, 27.
(3) Noguchi, H.: Jour. Exp. Med. 1909, **11**, 84.
(4) Kolmer, J. A., and Pearce, R. M.: Jour. Infect. Diseases, 1916, **18**, 32.
(5) Kolmer, J. A.: Jour. Infect. Diseases, 1916, **18**, 46.
(6) Noguchi, H.: Cited by Calmette.
(7) Calmette, A., Massol, L., and Breton, M.: C. R. Acad. des Sciences, 1908, **146**, 676.
(8) Calmette, A.: Berl. Klin. Woch., 1914, **51**, 496.
(9) Caulfeild, A. H.: Proc. Royal Soc., 1911, B., **84**, 373.
(10) Friedemann, W., and Herzfeld, E.: Berl. Klin. Woch., 1911, **48**, 2106.
(11) Suranyi, E.: Berl. Klin. Woch., 1912, **49**, 409.
(12) Jobling, J. W., and Petersen, W.: Jour. Exp. Med. 1914, **20**, 468.
(13) Rusznyak, S.: Deutsche Med. Woch., 1912, **38**, 168.
(14) Huntoon, F. M.: Jour. Immun., 1921, **6**, 117.
(15) Rowe, A. H.: Arch. Int. Med., 1916, **18**, 455.

(16) Kapsenberg, G.: Annales de L'Institut Pasteur, 1921, **35**, 648.
(17) Nishida, Y., and Petroff, S. A.: Am. Rev. Tuberculosis, 1920, **4**, 322.
(18) Calmette, A.: L'Infection Bacillaire et la Tuberculose, Paris, 1920, 501.
(19) Renaux, E.: Compt. Rend. Soc. de Biol., 1920, **83**, 1299.
(20) Calmette, A.: L'Infection Bacillaire et la Tuberculose, Paris, 1920, 495.
(21) Caulfeild, A. H.: Jour. Med. Research, 1911, **24**, 101–212.

THE TOXICITY OF ACIDS FOR LEUCOCYTES, AS INDICATED BY THE TROPIN REACTION[1]

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INTRODUCTION

This study was undertaken primarily to obtain definite knowledge concerning those factors which prevent phagocytosis *in vitro.* In an earlier paper it was shown that the phagocytic test may be used successfully for the quantitative determination of immune bodies in antimeningococcus serum when the serological grouping of meningococci is taken into consideration. It happened occasionally, however, that negative results were obtained for all the serums (including the positive control) on certain days. It appeared that the causes for such results were probably the same as have led immunologists generally to condemn the phagocytic test as unreliable. With the information obtained during the course of this study it is now possible to carry out quantitative tests with assurance that results will be dependable. It appears that the sensitiveness of leucocytes to acids was responsible for at least a part of the former occasional failures of the test.

Although the investigation was undertaken for practical purposes, it led to a consideration of the possibility, which has been suggested in the literature, that the changes in the blood in cases of lowered alkaline reserve may influence the activity of leucocytes, and thereby affect the course of infectious diseases.

REVIEW OF LITERATURE

Influence of H-ions on chemotropism. The studies which relate to the influence of H-ions on chemotropism in the lower animal and

[1] Approved for publication by the Surgeon General.

vegetable forms are of interest, because of the similarity between leucocytes and the motile microörganisms.

The phenomenon of chemotaxis was first investigated by Pfeffer. He discovered that the spermatozoa of ferns are strongly attracted by malic acid and its salts. A few years later Buller confirmed Pfeffer's observation. He found, however, that free malic acid attracted the spermatozoa only when in dilute solutions whereas it repelled them in solutions containing 0.03 gram per cent or more. He found that sodium malate attracted the spermatozoa in solutions isomolecular with malic acid solutions strong enough to repel them. As a result of his observations Buller advanced the hypothesis that the H-ion is the cause of repulsion by acids. Shibata investigated the behavior of malic and other acids toward the spermatozoa of Isoetes. He found that there were two agents in malic acid active toward these spermatozoa. The anion attracted and the H-ion repelled them. In relatively weak concentrations (1/500 mol.) the attraction of the malate ion was overcome by the repulsion of the H-ion. Shibata made the observation that sensitiveness to H-ions appears to be a rather general property of motile microörganisms. Bruchmann found that free citric acid in weak solution and the salts of citric acid attract the spermatozoa of Lycopodium into the archegonium during the process of fertilization. He found, however, that a 0.1 per cent solution of the acid did not give the favorable results obtained with the same strength of solution of the salts of citric acid.

Jennings found that acids were injurious to Paramoecia. He did not attribute the harmful effect to the H-ion, but to the anion.

Garrey studied the effect of various ions on Chilomonas. He found that these infusoria showed "chemokinesis" toward hydrochloric, nitric, and sulphuric acids in N/1000 dilution, and sometimes in dilutions as great as N/3000. He applied the term chemokinesis to a movement into areas of less concentration of the test substance. Since in these solutions the acids were completely dissociated and the anions produced no effect, he concluded that the effects produced by the acids were due to the H-ions. With solutions of organic acids he found conditions more complex. Dissociation of the organic acids was not complete, and the undissociated molecules were considered to play some part in the various phemonena observed when the solutions were allowed to act on cultures of Chilomonas. The organism was found to be chemokinetic to all the organic acids tested. On the other hand it was shown to be positively chemotropic to acetic, lactic, and butyric acids

when these were offered in very weak solutions. Garrey believed that the chemotropism was due to the anion, or to the undissociated molecules.

Barratt also studied the effect of acids on Paramecia. He found that they were indifferent to weak solutions of acids, but that they showed significant negative chemotaxis towards lethal solutions.

Koltzoff studied the action of H-ions on the phagocytosis of Carchesium lachmani, a fresh water infusorium. His experiments were carried out in distilled water, with India ink as "food." He found that a H-ion content of pH 4.87 interfered with phagocytosis, and phagocytosis was completely inhibited at pH 4. He concluded also that inhibition of phagocytosis by no means signified the death of the infusoria, for when they were replaced from an acid solution into distilled water they regained their capacity for engulfing the particles.

The effect of acid on leucocytes. The authors quoted above have agreed in showing that acids exert an inhibition of chemotropism on the various motile animal and vegetable organisms studied. Several investigators have studied the effect of acid on leucocytes.

Gabritchevsky placed under the skin of frogs and rabbits capillary tubes closed at one end and filled with the substance to be tested. By counting the number of leucocytes which wandered into the tubes he judged whether the substance was positively or negatively chemotactic, or indifferent. Among the substances which he listed as exerting negative chemotaxis was lactic acid in all concentrations.

Hamburger and Hekma studied the influence of various ions on phagocytosis, and found that the activity of leucocytes was decreased when N/2 sulphuric acid was added to the blood serum in which they were suspended in such quantity that a N/600 dilution of the acid was obtained. They found that the addition of similar quantities of acid to salt solution had a very much more pronounced unfavorable influence. Hamburger reported further experiments on phagocytosis in which horse leucocytes were tested with carbon particles as food. One volume of blood containing the leucocytes was diluted with 9 volumes of the test substance. Butyric acid in 1 to 100,000 dilution prevented phagocytosis notably; in 1 to 500,000 dilution it increased phagocytosis. Propionic acid in 1 to 500,000 dilution injured phagocytic activity; in dilutions of 1 in 1,000,000 to 1 in 5,000,000 it accelerated phagocytosis.

Schwyzer limited his observations to the pseudopodia formation of leucocytes under the influence of varying H-ion concentrations. He found that the optimum concentration for leucocytic activity was about

the neutral point. With increasing H-ion concentrations the leucocytic activity diminished.

In a recent paper Wolf reported a study of the influence of various chemicals on chemotaxis of leucocytes. She found that lactic acid was negatively chemotactic. She believed the negative influence was due in part to the H-ions, and that the lactate ion also had a negative influence.

According to Wells, amebae and presumably leucocytes react to stimuli of various kinds chiefly through the effect of these stimuli on surface tension. If they decrease the surface tension, the cell goes toward them, if they increase the tension, the cell moves away. Schwyzer found that H-ions increase the surface tension of leucocytes. Wells believes that this may explain the fact that lactic and other acids exhibit negative chemotaxis.

The influence of acid upon the opsonic reaction. In view of the fact that all those investigators who studied the effect of acid on various species of microörganisms found that it inhibits phagocytosis, and that those investigators quoted above who studied the general effect of acid on the activity of leucocytes obtained results in agreement with the botanists and zoölogists, it is rather remarkable that those who have studied phagocytosis as an immunological process have generally ignored the sensitiveness of leucocytes to acid or have reported contradictory results.

Three independent investigators, Noguchi, Eggers, and Arkin, tested the effect of acids on the opsonic reaction of normal serum and found it inhibitive. They all concluded that the opsonins are sensitive to acid, and the quantitative experiments of Brooks on the effect of H ions on complement (the thermolabile element of opsonin) have confirmed their conclusion. The authors quoted, did not, however, consider the effect of the acid on the leucocytes.

Bechhold, and Schütze both reported that phagocytosis is promoted by dilute solutions of acid.

Irala found that lactic acid in concentrations of 0.5 to 0.05 per cent added to defibrinated blood inhibited phagocytosis.

Sawchenko and Aristowsky found a neutral or feebly acid reaction the optimum for phagocytosis of red blood cells; for phagocytosis of *B. typhosus* an alkaline reaction was more favorable; whereas for phagocytosis of the cholera vibrio the greatest activity was found in a distinctly acid medium.

Oker-Blum found a neutral reaction most favorable for phagocytosis, but he reported that if the leucocytes were previously treated with dilute solutions of acid there was an enhanced phagocytic activity.

Relationship between blood reaction and phagocytosis in vivo. References are occasionally found in which the possibility is considered of relationship between the alkaline reserve in the fluids of the body and its ability to combat an infecting organism by means of phagocytosis.

In connection with his results showing that acid inhibits phagocytosis, Hamburger states that it has been obverved that if the alkaline content of the blood serum decreases in cases of infectious disease the prognosis is unfavorable, but if it begins to increase recovery is expected.

Dragstedt studied the blood reactions of rabbits which were infected with *Streptococcus hemolyticus* and found such slight variations from the normal that he believed the development of acidosis in an acute infection could play no rôle in checking the growth of bacteria; but he suggested that an alteration in the reaction of the body fluids may influence the fixation of antigens or the activity of the leucocytes.

Otsubo reported that he reduced the alkaline reserve of the blood of guinea pigs by repeated injections of various salts. He then injected streptococci into the abdominal cavity and made smears of the peritoneal fluid three hours later. He correlated a reduction of phagocytic power with a diminution in the alkaline reserve of the blood.

Those who support the contention that the blood reactions in acidosis may have an unfavorable influence on phagocytosis quote the results of those investigators who have noted a lowered resistance of the blood to infection accompanying a decreased alkaline reserve. Von Behring was the first to make this observation. There will be no attempt made to list the many investigators who have confirmed von Behring's observations. Attention is called to the fact, however, that von Behring and those who have confirmed his observations were considering the bacteriolytic power of the serum from which the leucocytes had been removed. Their results are not applicable to the relation of H-ion concentration of the blood to the phagocytic power of leucocytes.

Ranges of H-ion concentration of blood plasma and other body fluids. In view of the hypothesis that the H-ion concentration of the body fluids may influence phagocytosis, it is of interest to summarize the literature dealing with the variations in pH values of the body fluids which may occur during an infection.

The work of Henderson, Van Slyke and Cullen, Bayliss, and others shows that the H-ion concentration of the blood is remarkably constant. Summarizing the available knowledge on the subject Van Slyke in a recent paper states that under extreme abnormal conditions the pH may fall as low as 6.95, but before this point is reached coma occurs. The extreme range of reaction compatible with life lies between pH 7.0 and 7.8; the normal range lies between pH 7.3 and 7.5, possibly within narrower limits.

Wright tested the alkaline reserve of the blood and of the lymph in the infected tissues of patients with gas gangrene. He found the average blood "alkalinity" of normal men was N/30 to N/35; that of 15 gas gangrene patients was N/55. He reports one case in which the "alkalinity" of the lymph in the infected tissue was N/200. The "alkalinity" of the blood at that time was N/70. The patient recovered. In another case the "alkalinity" of the pleural effusion was N/100 whereas that of the blood was N/50. These data are not directly applicable to the problem under consideration, because Wright expressed acidosis in terms of the alkaline reserve, instead of in terms of H-ion concentration, but they indicate that acidosis may be more pronounced in the infected tissue than in the body as a whole.

According to Wells the reaction of pus serum is usually slightly alkaline, but sometimes lipase derived from bacteria or from the cells causes splitting of sufficient amounts of fatty acids from the fats to make the reaction acid, and lactic and other fatty acids are formed, depending on the nature of the infecting organism. Wells does not give figures for these reactions.

Judging from the data given by Wright and the statement made by Wells it would appear that there may be somewhat greater variations in the pH of the fluids in infected tissues than in those of the general circulation. Van Slyke states, however, that the limited knowledge on this subject indicates that the body fluids other than blood plasma approximate the latter closely in their reaction.

The buffer action of living cells. No references relating to the absorption of H-ions by leucocytes could be found in the literature.

Using the eggs of trout Gray considered the absorption of H-ions by living cells. He found that when normal eggs were exposed to weak acid solutions they effected a decrease of H-ions. He found that the total amount of H-ions removed from a solution depended upon their concentration in the original solution. He states that all the results of his experiments are explained if it is assumed that K-ions or NH_3-ions leave the egg and H-ions take their places.

The extraordinary buffer action of red blood cells for H-ions has been shown by Haggard and Henderson, and by C. L. Evans. Their results are of interest in connection with this study, but they are not directly applicable since these investigators believe that the buffer action of red blood cells is due to their hemoglobin.

The effect of various acids on living cells. A number of investigators have shown that organic acids have more pronounced action on living cells than have inorganic acids of the same H-ion concentration. Loeb found differences in the behavior of muscle toward organic and inorganic acids. He found that a muscle in 0.7 per cent sodium chloride solution did not change its weight in an hour, but if a trace of acid was added, there was a significant increase in weight. The action of the inorganic acids was in exact proportion to the number of H-ions in a unit volume of the solution, but no such relationship was apparent for the organic acids. Lactic and mandelic acid caused more water to be taken up in proportion to their degree of dissociation than the other acids tested.

Richards and Kahlenberg at about the same time reported that solutions of mineral acids of corresponding H-ion concentration gave a uniformly sour taste, but that acetic acid was found to be several times as sour as would be expected if the taste was due to the H-ions momentarily present. Neither of these investigators offered an explanation for their observations.

Garrey's work has already been mentioned. He found that the inorganic acids showed the same effects on Chilomonas if they contained the same number of H-ions in a unit volume of solution, but the organic acids did not show a definite relation between the number of H-ions and the effects produced. Acetic and lactic acids were more than three times as active as they should be if H-ions were the only factor in the reaction.

In his experiments on artificial parthenogenesis and fertilization Loeb found that a short exposure of the eggs of the sea-urchin to a cytolytic agent led to the formation of a typical fertilization membrane; a longer exposure entailed cytolysis. If, however, after a short exposure to a cytolytic agent the eggs were subjected to a short exposure to hypertonic sea water they could be induced to develop into larvae. Carbonic acid and the weak mono-basic fatty acids were very effective in inducing membrane formation, whereas the strong mineral acids were much less effective. Those acids which were most effective for membrane formation were also the most toxic to the eggs if they were given a too long exposure. Loeb is of the opinion that the acids enter

the egg in the form of undissociated molecules, and that their physiological action is due to their velocity of diffusion into the egg. He found that the effect of the acid increases with the number of carbon atoms; that an OH group has the opposite effect; and that the "linear" coupling of the carbon atoms is more effective than the branched.

Harvey studied the relative permeability of the cells of Stichopus ananas (prickly fish) for various acids. He found that degree of dissociation was not the determining factor in penetration but that there was a certain correspondence between lipoid solubility and capillary activity and penetration rate. He believed therefore that there was more than one variable concerned in determining the rate of penetration, and that the facts could be explained by assuming that the cells are composed largely of fat-protein combinations in which the visible physical characteristics of fat are masked.

Harvey studied also the toxicity of various acids for the cilia of the giant clam. He found no relation between toxicity and dissociation. He compared his own results with the results of Barratt, Fühner and Neubauer, Kahlenberg and True, and Loeb (1898, 1909, 1913). All these investigators had studied the effect of various acids on living cells. Harvey expected to find that the different organisms would vary in resistance to the same acid, but that the series of acids arranged in order of efficiency in affecting various tissues would be the same. Contrary to his expectations, he found no agreement between the series.

Crozier studied the rate of penetration of acids into the tissue of a nudibranch, Chromodoris zebra. His results showed the same order of efficiency as in the case of Harvey's experiments. Crozier concluded that ionization is the primary determinant of cell penetrating power, but that there is some other factor controlling entrance into the cell, which he believes is related to lipoid solubility.

Haas used the petals of Browallia and the Hyacinth perianth to study the permiability of living cells to acids. The rate of penetration of the various acids into these plant tissues coincided with the results obtained by Harvey and Crozier with animal tissues.

Winslow and Lochridge found that the toxicity of hydrochloric and sulphuric acid solutions for *B. typhosus* and *B. coli* depended on the number of dissociated H-ions. Results similar to those obtained with the mineral acids were obtained with acetic acid in one-sixth the strength of ionic hydrogen. They concluded that the toxicity of the organic acids tested was due mainly to the action of the undissociated molecule.

Wolf and Harris investigated the effect of acids on the growth of pathogenic anaerobes. They did not observe a distinctly specific toxicity of any acid, but they found a definite effect proportional to the common cation, the H-ion. These investigators believed that the anions, and the undissociated acids behaved merely as foreign bodies, possessing a certain inhibiting effect proportional to their molecular concentration.

On the other hand Wyeth found a small but definite result on the growth of *B. coli* due to the specificity of action of the various added acids, although, when the mass of acid introduced was considered, the strong acids were more inhibitory than the weaker or less dissociated acids.

Cohen and Clark found that the critical pH zone inhibiting the growth of *B. coli* was 5.5–5.7 when the medium was acidified with acetic whereas it was 4.6–5 in the case of hydrochloric acid.

A general summary of the literature reviewed above leads to the following conclusions: (1) Living cells absorb H-ions from weakly acid solutions. (2) An acid reaction of the medium interferes with phagocytosis in simple animal and vegetable organisms. (3) Organic acids possess some property by which the various effects of H-ions characteristic for the numerous kinds of living cells with which experiments have been conducted are more pronounced than can be explained by their degree of dissociation.

In making a digest of the results of those investigators who have studied the effect of acid on the phagocytic reaction as applied to serological tests, it is more difficult to draw definite conclusions. That the results of different investigators have not agreed is not surprising when two conditions of their experimentation are considered. First, many of the investigations were made before the development of the colorimetric method for the determination of H-ion concentrations, by which fine distinctions may be readily made. Secondly, all these investigators studied spontaneous phagocytosis of inert particles, or phagocytosis promoted by the antibodies present in normal serum (opsonins). Phagocytosis under those conditions is not vigorous. The difference between a positive and negative reaction is so slight that experimental errors may easily confuse the interpretation of results.

In the experiments recorded in this paper quantitative determinations of the H-ion concentration of the solutions were made by the colorimetric method. The "food" for the leucocytes was bacteria sensitized with an immune serum which promoted such a marked degree of phagocytosis that there could be no question in distinguishing between a positive and a negative reaction. The immune body utilized for sensitization was that stable body which is generally designated as bacteriotropin. Its stability as compared with the opsonins of normal serum, which act only in the presence of labile complement, is remarkable. The bacteriotropins withstand inactivating temperatures (Evans 1920), light (unpublished data), acid reactions (see experiment 4 of this paper) and age. Many of the experiments here recorded were done after the serum was a year and a half old. It retained the immune body in abundance. In the later experiments a 1 to 500 dilution of serum was used to sensitize a suspension of cocci of a density equal to 6000 parts per million (roughly estimated, 12,000,000,000 organisms per cubic centimeter).

DESCRIPTION OF METHODS

The technic used was that originally used by Neufeld, with certain modifications.

In the early experiments the diluent for serum dilutions, bacterial antigens, and leucocyte suspensions was 0.85 per cent sodium chloride solution. During the progress of this study a buffered saline solution was devised and used as a diluent in all the later experiments. The advantages of buffered solutions for serological work in general were pointed out in a previous paper (Evans, 1921). Briefly, the buffered solutions were prepared by adding 1 part of Sorensen's phosphate buffer mixture, adjusted to the reaction desired for the experiment, to 9 parts of a 0.9 per cent sodium chloride solution. In this paper the buffered medium will be referred to as "buffered saline solution," to distinguish it from the 0.85 per cent sodium chloride solution, which will be designated "physiological saline solution."

With the exception of the one experiment for which the protocol is given in table 1, the serum used in these tests was always the same. It was an anti-streptococcus rabbit serum of high titer produced by

repeated injections with increasing doses[2] of a strain of *Streptococcus viridans* (no. 344) originally obtained from a case of rheumatism. The serum contained tropins in the 1 to 36,000 dilution. A dilution of 1 to 5000 was always used in the early experiments. In the later experiments higher concentrations of serum were used, but the sensitized bacteria were washed free from the serum before the addition of leucocytes. Thus the tests were freed from the complication of a buffer action of the serum.

The bacterial antigens were prepared from twenty-four hour broth cultures of the strain used for the production of the serum. Whenever glucose was added to the broth, it was in 1 per cent concentration. The cultures were centrifugalized and the sediment was taken up in a small quantity of saline solution. The turbidity of this heavy suspension was determined by comparison with the silica turbidity standard adopted by the American Public Health Association. A vial was filled with the silica standard diluted to 300 parts per million. Letters of a certain chosen type were found to be just legible when read through this vial. A measured quantity—usually 0.2 cc.—of the dense bacterial suspension was placed in a vial of a size and shape similar to that containing the standard and diluted to match the density of the silica standard. The bacterial antigen to be used in the test was then prepared with a density equal to twice the density of the standard (the addition of an equal quantity of serum dilution gave a suspension of the proper density); or, if the antigen was washed after sensitization, it was made up to a density of 300 parts per million.

Two-tenths of a cubic centimeter of serum dilution and an equal quantity of bacterial suspension were placed together in 10 mm. by 75 mm. reagent tubes, and incubated in a 37°C. water bath for 45 minutes. During the incubation the leucocytic suspension was prepared. (In the later experiments 0.4 cc. of washed sensitized antigen were placed in each test tube.)

Rabbit leucocytes were used.. They were usually obtained by injecting into each pleural cavity about 5 cc. of sterile aleuronat[3] suspension on the day preceding the test. Aleuronat was not always available, however, and a 5 per cent peptone solution was used as a substitute with good results.

[2] The first dose was 2 cc. of a twenty-four-hour killed broth culture. The eighth (last) dose was the growth from 10 cc. of living broth culture, suspended in 2 cc. of normal saline solution.

[3] The aleuronat suspension was made by adding 3 per cent starch and 5 per cent aleuronat to ordinary broth.

All solutions in which the leucocytes were to be suspended were warmed to 37°C. The exudate was taken up in a 1 per cent solution of sodium citrate in physiological saline solution. About 50 cc. of the citrate solution were used for washing each pleural cavitv. If the exudate was found to be very bloody the first fractions of the bloody washings were discarded and the later fractions were usually found to be sufficiently free from red blood corpuscles to be used in the test. Usually small particles of aleuronat or small clots of fibrin or blood were washed out with the exudate. They sank to the bottom of the container and were disposed of by decanting the supernatant suspension into 50 cc. centrifuge tubes. The leucocyte suspension was centrifugalized for four minutes at such a speed that the majority of leucocytes were thrown to the bottom of the tube, leaving a slightly clouded supernatant fluid (the cloudiness indicating that the leucocytes had not been subjected to a compression great enough to injure them). The supernatant fluid was poured away and the sediment was uniformly suspended in about 50 cc. of saline solution per tube. The suspension was centrifugalized the second time in the same manner as before. The sediment was then carefully emulsified in saline solution, allowing about 7 cc. for the leucocytes from each cavity.

It was found to be impracticable to standardize accurately the density of the leucycyte suspension, because there was commonly a variable quantity of red blood corpuscles mixed with leucocytes in the exudate. In small quantities the red cells did not interfere with the phagocytic reaction, but they did interfere with the standardization of the density of the leucocyte suspension by the transparency test. Therefore the only standard adopted for the density of the leucocytic suspension was a uniform method of procuring the leucocytes.

After the forty-five minutes' incubation for the sensitization of the antigen, 0.2 cc. of leucocyte suspension were added to each of the tubes. They were shaken to obtain a uniform suspension, and then returned to the water bath for further incubation. During the second incubation period the racks containing the test tubes were kept in vigorous motion by an electric shaking apparatus, in order to prevent the leucocytes from sinking to the bottom of the tubes. After forty-five minutes incubation the tubes were removed from the water bath and smears were made. Before making a smear a uniform suspension was obtained by vigorously rolling the tube between the hands. After drying, the smears were fixed with methyl alcohol.

They were allowed to dry again and then were stained for twelve seconds with a weak solution of Bordet-Gengou's toluidin blue.[4]

Phagocytosis by the polymorphonuclear leucocytes alone was considered in this study. A characteristic picture of the phagocytosis of bacteria which have been sensitized with immune serum shows a large percentage of those leucocytes which participate in phagocytosis to be crowded full of bacteria. For this reason it was found impossible to count the number of cocci ingested by the leucocytes, as is commonly done in the opsonic test. Twenty-five polymorphonuclear leuocytes in each smear were examined, and the presence or absence of bacteria was recorded in the terms of the percentage of leucocytes containing bacteria. It was observed that those leucocytes which were agglutinated generally contained more bacteria than the isolated leucocytes; an observation which has also been recorded by Fenn. Therefore, if there had been a clumping of leucocytes, one-half of the number counted was chosen from one or more groups and the remainder were chosen from the isolated leucocytes. Record was kept of the percentage of leucocytes containing more than 10 cocci. They were tabulated in terms of leucocytes "filled" with bacteria.

H-ion determinations were made with the use of the standard solutions and the indicators described by Clark and Lubs. Whenever the buffer action of material in suspension was under consideration, it was removed by centrifugalization and the determinations were made on the clear fluid.

EXPERIMENTAL WORK

Effect on the tropin reaction of producing the antigen in glucose broth culture

Experiment 1. The serum used in this test was from a rabbit which had received repeated injections of a culture of *Streptococcus viridans* (no. 328). The antigens were homologous to the serum. No effort was made to remove the medium from the sediment obtained by centrifugalization of the cultures. The sediment was emulsified in about one cubic centimeter of the supernatant broth, and diluted with physiological saline solution to the required density, as might be done

[4] Bordet-Gengou's toluidin blue is made by dissolving 5 grams of toluidin blue in 100 cc. alcohol, 500 cc. water and 500 cc. of 5 per cent phenol, and filtering after one or two hours. One part of stain was diluted with 2 parts of water for staining the smears.

in any experiment in which the reaction is ignored. The results are given in table 1. The antigen grown in ordinary broth gave a strongly positive reaction in a dilution of 1 to 600. On the other hand the antigen grown in dextrose broth gave a negative reaction in the lowest dilution tested (1 to 50).

It is plainly evident from the data presented in table 1 that it is hazardous to the test to grow the antigen in glucose broth. On account of the scant growth of streptotocci in ordinary broth, however, it would be advantageous to use glucose broth for the production of streptococcus antigens if that could be done without sacrificing the reliability of the test. Further tests were therefore carried out to show more definitely the sensitiveness of leucocytes to the toxic substances in glucose broth cultures.

TABLE 1

Phagocytosis is inhibited by the toxic products in glucose broth cultures

	SERUM DILUTIONS				
	50	100	200	400	600
Antigen grown in ordinary broth					76* 28†
Antigen grown in glucose broth	0	4 0	8 0		

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

Experiments 2 and 3. A number of bacterial suspensions were made up with increasing proportions of the broth in which the culture was grown added to the physiological saline solution used for diluent. The concentration of the H-ions in such a mixture forms a measurably toxic condition. Hence the gradations were expressed in terms of the H-ion concentration of the bacterial suspensions. The data for the two experiments are presented in table 2. The reaction of the medium was modified by the buffer action of the leucocytic suspension, as shown in the second and fifth columns. In experiment 2 an inhibition was evident when the H-ion concentration of the bacterial suspension was equivalent to pH 5; in experiment 3 an inhibition was evident when the pH of the bacterial suspension was 5.3. In both cases the given pH values were obtained when the proportion of acid broth to

physiological saline solution was 2 in 21. In both experiments there was complete inhibition of phagocytosis when the bacterial suspension had a pH value of 4.9. In experiment 2 this was obtained by adding

TABLE 2

Phagocytosis is inhibited by toxic substances from the glucose broth in which the antigen was produced

EXPERIMENT 2			EXPERIMENT 3		
pH of bacterial suspension	pH of bacteria-leucocyte suspension	Phagocytosis	pH of bacterial suspension	pH bacteria-leucocyte suspension	Phagocytosis
7.1*	7.0	76‡ 48§	7.1*	6.7	84 64
6.8†	6.9	88 36	6.5†	6.9	68 60
6.1	6.6	64 52	6.1	6.5	80 72
5.5	6.5	76 64	5.7	6.2	84 80
5.3	6.2	84 68	5.3	6.1	36 32
5.0	6.2	36 16	5.1	6.0	24 16
4.9	5.6	0 0	5.0	5.8	12 4
			4.9	5.6	0 0

* Control. Antigen was produced in plain broth.

† Control. Antigen was produced in glucose broth and washed in normal saline solution.

‡ The upper figure indicates the percentage of phagocyting leucocytes.

§ The lower figure indicates the percentage of leucocytes containing 10 or more cocci.

acid broth to saline solution in the proportion of 5 to 21; in the third experiment it was obtained by adding acid broth to saline solution in the proportion of 7 to 21.

Experiment 4. It has been noted that investigators have shown that sensitization of bacteria by the opsonins of normal serum is inhibited in acid solutions because the labile element (complement) is injured by the acid. No record was found of experiments showing the effect of acid on the activity of bacteriotropins. In table 3 is given the proto-

TABLE 3

The activity of leucocytes is inhibited, but the union of bacterial cells and antibody is unaffected by an acid medium

	pH of bacterial suspension	pH of original serum-bacteria suspension	pH of final sensitized bacteria suspension	pH of bacteria-leucocyte suspension	Phagocytosis
1. Antigen A*. Regular routine procedure. Control test.	6.8	6.8	6.8	6.6	68† 40‡
2. Antigen B.§ Regular routine procedure.	4.9	5.0	5.2	5.4	4 0
3. Antigen A. The bacterial suspension was sensitized in neutral medium, centrifugalized, and resuspended in normal saline solution	6.8	6.8	6.8	6.6	64 44
4. Antigen A. The bacterial suspension was sensitized in neutral medium, centrifugalized, and resuspended in acid medium.‖	6.8	6.8	5.2	5.6	0 0
5. Antigen B. The bacterial suspension was sensitized in acid medium, centrifugalized, and resuspended in normal saline solution.	4.9	5.0	6.5	6.6	76 36

* Antigen A was prepared from a plain broth culture. The culture was centrifugalized, the supernatant fluid was removed, and the antigen was suspended in normal saline solution.

† The upper figure refers to the percentage of phagocyting leucocytes.

‡ The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

§ Antigen B was prepared from a glucose broth culture. The culture was centrifugalized and resuspended in 1 part of the supernatant fluid and 1.5 parts of the acid medium.

‖ The acid medium was prepared by adding 5 parts of normal saline solution to 1 part of the supernatant fluid obtained by centrifugalizing the glucose broth culture from which Antigen B was prepared.

col of an experiment planned to show whether the toxic substances in glucose broth culture interfere with the sensitization of bacteria, or with the activity of the leucocytes, or both.

The results of the experiment clearly indicate that the acid reaction of the serum-bacterial suspension did not interfere with the sensitization of the bacteria, for when the acid medium in which the sensitization took place was replaced by physiological saline solution (test 5) phagocytosis was as vigorous as when the sensitization took place in a neutral medium (tests 1 and 3). But phagocytosis was completely inhibited when the final bacteria-leucocyte suspension was in the acid medium (tests 2 and 4).

When carrying out the phagocytic test with streptococci it is practicable to grow the antigen in glucose broth and prepare it according to the following procedure which was used in experiments 8 to 14. After centrifugalization the supernatant fluid was removed as completely as possible, then the sediment was washed with a small quantity of buffered saline solution which was allowed to flow down the side of the centrifuge tube without disturbing the sediment. This fluid was removed as completely as possible, and the sediment was then taken up in buffered saline solution to make an antigen of the proper density.

Effect on leucocytes of acid sodium citrate solutions

Another observation of injury to leucocytes in acid solutions was made when carrying out the test with meningococci, which ferment sugars so slowly that the production of acid by the antigen does not need to be considered.

It happened on several occasions that poor tropin reactions were obtained with serums known to have a good content of antibodies. The explanation was not at first apparent, for all solutions used in carrying out the tests had been made up as usual, and, so far as observation could go, the test proceeded in the ordinary manner without mishaps. On testing the sodium citrate solution used to take up the leucocytic exudate one day when negative results had been obtained it was found to have a H-ion concentration of pH 5.6. It seemed possible that the acid reaction of the sodium citrate solution had interfered with phagocytosis. Several bottles of sodium citrate prepared by three different manufacturing firms were examined. All of the bottles were labeled "$2\ Na_3C_6H_5O_7 \cdot 11H_2O$. C.P." However, there was a great difference between the pH values of solutions prepared from them, no two solutions

being found alike. The pH values varied between 5.4 and 8.7. One bottle prepared by each of the three different firms was examined more carefully. Individual crystals were picked out and H-ion determinations were made on the solutions prepared from them. Each of the

TABLE 4
Leucocytes are injured by acid sodium citrate

EXPERIMENT NUMBER	TEST NUMBER	pH VALUE OF ANTIGEN	pH VALUE OF CITRATE SOLUTION = 8.7	pH VALUE OF CITRATE SOLUTION = 5.6
5	1	6.5	68*	40
			60†	32
	2	6.1	84	24
			80	20
6	3	7.1	68	56
			52	28
7	4	7.1	76	68
			48	56
	5	6.8	88	52
			36	36
	6	6.1	64	44
			52	24
	7	5.5	76	16
			64	8
	8	5.5	80	24
			44	12
	9	5.3	84	24
			68	4
	10	5.0	36	0
			16	0

* The upper figure refers to the percentage of phagocyting leucocytes.
† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

bottles was found to contain a mixture of the secondary (acid) and tertiary (alkaline) salts in varying proportions, and of varying purity.

Experiments 5, 6 and 7. Controlled experiments were carried out to show the effect of taking up the leucocytic exudate in sodium citrate

solution (one per cent sodium citrate in physiological saline solution) of a pH value of 5.6. The results of several such experiments are given in table 4. The tests are numbered 1 to 10. The only variable factor in a given test was the taking up of the leucocytic exudate in a sodium citrate solution of pH 5.6 or 8.7, respectively. By comparing the results shown in the last two columns of the table, it may be noted that a lower percentage of phagocytosis was usually obtained when the leucocytic exudate was taken up in the acid citrate. The extent of the reduction of phagocytosis brought about by the use of the acid citrate varied greatly, however. When the H-ion concentration of the final bacteria-leucocyte suspension was favorable, the previous injury to the leucocytes by the acid citrate was not marked (tests 1, 3, 4, and 5). When, however, the H-ion concentration of the bacterial suspension to which the leucocytes were added approached the point of toxicity, the leucocytes which had been previously exposed to the acid citrate were definitely more impeded in their activity than were those leucocytes previously uninjured (tests 2,6 to 10).

Optimum H-ion concentration for phagocytosis

Experiment 8. This experiment was planned to show whether the reaction of rabbit's blood, which according to Dragstadt is about pH

TABLE 5

Triplicate tests show that phagocytic activity is practically the same in saline solution of pH 7 and 7.6

pH OF SALINE SOLUTION	PHAGOCYTIC ACTIVITY		
	(1)	(2)	(3)
7.0	80* 56†	76 48	76 44
7.6	76 24	68 44	72 44

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

7.6, is more favorable for phagocytic activity than a neutral reaction. The protocol is given in table 5. The data show no significant difference in phagocytic activity at the two reactions tested. In the following experiments a H-ion concentration equal to pH 7 was adopted for the standard solution.

Relative toxicity of various acids

Experiment 9. This was planned to show the relative toxicity of citric, lactic and acetic acids, as compared with hydrochloric acid on the basis of equivalent H-ion concentration. The results are presented in table 6. Buffered saline solution was acidified by the addition of the various acids, adjusting the H-ion concentrations of the solutions to pH 4.6 and 4. Equal quantities of leucocytes were washed with 10 cc. of the various test solutions. After centrifugation, the test solutions were washed away and the final suspension was made in buffered

TABLE 6

The relative toxicity of the various acids is in the order in which they appear in the column

	LEUCOCYTES WASHED WITH SALINE SOLUTION OF pH		
	7.0	4.6	4.0
Control	76* 56†		
Hydrochloric			76 60
Citric		72 68	76 44
Lactic		64 28	0
Acetic		20 4	4 0

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

saline solution of pH 7. The sensitized antigen was washed free of serum and suspended in buffered saline solution of pH 7. The various acids are arranged in the table in the order of their relative toxicity. The leucocytes treated with the hydrochloric and citric acids showed no injury under the conditions of this experiment.

Experiment 10. This is a repetition of experiment 9 confirming the results of that experiment. The results of experiment 10 are given in table 7. In addition to an attempted confirmation of experiment 9, this experiment was designed to show whether the toxic solutions under consideration would be rendered innocuous by neutralization.

The leucocytes washed with hydrochloric and citric acids showed no injury; those washed with lactic acid showed partial inhibition of phagocytosis; and those washed with acetic acid showed complete inhibition. The results obtained were therefore in agreement with those obtained in experiment 9. Lactic acid is shown to be distinctly more toxic than hydrochloric and citric, and acetic acid is shown to be more toxic than lactic acid of equivalent H-ion concentration.

TABLE 7

The relative toxicity of the various acids is in the order in which they appear in the column. Toxic acid solutions become non-toxic when neutralized with NaOH

	Leucocytes washed with buffered saline solution of pH 7.0	Leucocytes washed with buffered saline solution acidified to pH 4.0	Leucocytes washed with the same solutions readjusted from pH 4.0 to pH 7.0 by the addition of NaOH
Control (1)	36* 32†		
Control (2)	44 28		
Hydrochloric		44 28	32 28
Citric		40 20	44 20
Lactic		32 4	32 24
Acetic		4 0	40 24

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

The question may arise in the reader's mind as to why leucocytes treated with lactic acid pH 4 were injured so that phagocytosis was completely inhibited in experiment 9, but only partially inhibited in experiment 10. The reason is that in experiment 10, a greater number of leucocytes were exposed to a given quantity of acid than in experiment 9.

The leucocytes washed with solutions in which lactic and acetic acids had been neutralized with sodium hydroxide, showed no injury.

This observation was confirmed in another similar experiment for which the data are not given. The effect on leucocytes of the salts of organic acids is again considered in experiment 14.

Experiment 11. Experiments 9 and 10 did not demonstrate the relative toxicity of hydrochloric and citric acids, or indeed, whether they are toxic at all. Experiment 11 was planned to compare the effects of these two acids. The protocol is presented in table 8. The leucocytes were washed as in the previous experiments, and then suspended

TABLE 8

Citric acid is shown to be slightly (if any) more toxic than hydrochloric acid of similar H-ion concentrations

HYDROCHLORIC ACID					CITRIC ACID				
pH of antigen	Leucocytes washed with saline solution of pH				pH of antigen	Leucocytes washed with saline solution of pH			
	7.0	5.6	4.6	4.0		7.0	5.6	4.6	4.0
7.0	60* 32†	60 32	60 36	72 44	7.0	56 44	60 32	72 48	68 44
5.8	68 32	64 48	64 48	60 48	5.8	48 28	72 36	72 44	40 20
5.3	52 36	60 40	68 44	68 48	4.6	64 36	60 32	28 16	12 4
4.9	48 36	68 48	56 40	48 32	4.1	44 12	36 12	8 0	8 0

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

The figures showing inhibition of phagocytosis are indicated by the heavy line.

in buffered saline solution of pH 7. But in this experiment further variations were introduced by preparing series of antigens with the use of the same test solutions as were used for washing the leucocytes. Thus the leucocytes were twice exposed to acid solutions. Since the dissociation constants of the two acids differ considerably at the two higher concentrations of H-ions used in these experiments, the buffer action of the bacterial cells reduced the acidity of the hydrochloric acid solutions more than that of the citric acid solutions. Hence the antigen which was prepared with the HCl solution of pH 4 is more comparable

with the antigen prepared with the citric acid solution of pH 4.6 than with the antigen prepared with the corresponding citric acid solution. This fact must be considered in interpreting the results of the experiment.

The leucocytes subjected to the greatest amount of hydrochloric acid were scarcely injured by it. Those washed in the most highly acid solution and mixed with the most highly acid antigen gave slightly lower figures than the other tests, but the difference is not significant. The figures for all of the other tests of hydrochloric acid solutions are as nearly alike as would be expected in comparative tests if all conditions were the same, signifying that the leucocytes were uninjured by the acid solutions.

The leucocytes washed in citric acid solution of pH 4 and mixed with the antigen of pH 5.8 appear to have suffered some injury. Those washed in the citric acid solution of pH 4.6 and mixed with the antigen of pH 4.6 were definitely injured. The results of the experiment indicate that citric acid is slightly more toxic to leucocytes than hydrochloric acid of equivalent H-ion concentrations.

Experiment 12. The preceding experiments have not shown that hydrochloric acid is toxic for leucocytes. Experiment 12 was planned to demonstrate the toxicity of hydrochloric acid. The protocol is presented in table 9. The leucocytes were washed as in the preceding experiments, but instead of making the final leucocyte suspension in neutral saline solution, they were made in the same test solutions as were used for the washing. As in experiment 11, a series of antigens was made up in the various test solutions. The pH values of the antigens after the modification of the H-ion concentration of the solution by the buffer action of the bacterial cells is given in the table.

In this experiment, then, the test acid solutions were of pH values similar to those used in experiment 11, but the leucocytes were subjected to them once more than in that experiment. The toxicity of hydrochloric acid for leucocytes is well demonstrated. Since hydrochloric acid is completely dissociated in solutions of low concentration it can be assumed that the toxicity of this acid is due to its free H-ions.

Experiment 13. The toxicity for leucocytes of butyric acid, a common product of pathogenic bacteria, has not yet been considered. Preliminary experiments showed that it was of about the same toxicity as acetic acid, possibly a little more toxic. The protocol for a detailed experiment, similar to experiment 11 for showing the relative toxicity of hydrochloric and citric acids, is given in table 10.

Equal quantities of leucocytes were washed with the various test solutions, the test solutions were washed away, and then the leucocytes were suspended in buffered saline solution of pH 7. Antigens were prepared in the same test solutions. The dissociation constants of acetic and butyric acids are similar, hence the buffer action of the bacterial suspension had the same effect on the corresponding solutions of the two acids. It increased the pH value of both 5.6 solutions by 0.1. The 4.6 solutions were not perceptibly changed by the addition of the bacteria. The figures in the corresponding positions in the table are therefore exactly comparable. The data show very little difference in the toxicity of acetic and butyric acids. There is, however, a slight indication that butyric acid may be somewhat more toxic than acetic acid.

TABLE 9

The toxicity of hydrochloric acid for leucocytes

pH OF ANTIGEN	LEUCOCYTES WASHED WITH BUFFERED SALINE SOLUTION AND SUSPENDED IN THE SAME SOLUTION			
	pH of solutions			
	7.0	5.6	4.8	4.0
7.0	60* 60†	84 40	72 48	76 44
5.6	80 48	72 44	56 20	36 8
5.1	76 52	40 24	24 12	20 0
4.8	80 64	36 20	20 12	16 0

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

The figures showing inhibition of phagocytosis are indicated by the heavy line.

Experiment 14. This was planned to obtain data supplementary to that given in table 7, which showed that toxic acid solutions were not harmful to leucocytes after neutralization. The effect on phagocytosis of one per cent of sodium citrate in the final suspension is shown in the protocol presented in table 11. The test solution was a one per

TABLE 10

Butyric acid is perhaps slightly more toxic than acetic acid

ACETIC ACID				BUTYRIC ACID			
pH of antigen	Leucocytes washed with saline solution of pH			pH of antigen	Leucocytes washed with saline solution of pH		
	7.0	5.6	4.6		7.0	5.6	4.6
7.0	32* 32†	40 24	40 16	7.0	36 16	32 28	28 24
5.7	44 28	32 20	32 8	5.7	32 20	20 12	12 12
4.6	20 8	8 4	12 0	4.6	20 8	4 0	0

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

TABLE 11

Duplicate tests showing that one per cent concentration of sodium citrate of pH 7.0 does not inhibit phagocytosis

CONTROLS. FINAL SUSPENSION IN BUFFERED PHYSIOLOGICAL SALINE SOLUTION OF pH 7.0		FINAL SUSPENSION IN ONE PER CENT CITRATE SOLUTION OF pH 7.0	
64* 52†	64 44	68 44	80 64

* The upper figure refers to the percentage of phagocyting leucocytes.

† The lower figure refers to the percentage of leucocytes containing 10 or more cocci.

TABLE 12

Leucocytes absorb H-ions from weakly acid solutions

	pH BEFORE ADDITION OF LEUCOCYTES	pH AFTER ADDITION OF LEUCOCYTES			
		HCl	Lactic	Acetic	Butyric
Saline solution unbuffered	5.0 5.6	6.7 6.6		 6.8	
Saline solution buffered	4.0 4.8 5.6	5.0 5.4 5.7	4.5 5.1 5.7		
Saline solution buffered	4.0 4.6	4.8 5.2		4.6 4.7	4.6 4.7

cent solution of commercial alkaline sodium citrate in physiological saline solution. The reaction was adjusted to pH 7 by the addition of hydrochloric acid. The treatment of the washed sensitized bacteria and the leucocytes in the control suspensions and in the test suspensions was exactly the same, except for the different solutions used in making up the final suspensions. The differences in results, slightly in favor of the citrate solution, are within the limits of experimental error. Thus it is shown that a solution of sodium citrate in one per cent concentration having a H-ion concentration of pH 7 is not toxic for leucocytes. This result is of special interest, as it will be pointed out in the later discussion.

TABLE 13

A summary of the literature shows a similar order of effectiveness of various acids on various kinds of living cells. The effectiveness increases in the descending column

TOXICITY TO CILIA OF GIANT CLAM*	PENETRATION OF BROWALLIA PETALS†	TOXICITY TO B. COLI‡	TOXICITY TO LEUCOCYTES
HCl } Citric }	HCl Citric	HCl }	HCl Citric
Lactic	Lactic	Lactic	Lactic
Acetic } Butyric }	Acetic	Acetic }	Acetic Butyric

* Harvey, 1914.
† Haas, 1916.
‡ Wyeth, 1917.

DISCUSSION

General conclusions

Incidentally in connection with experiments designed to obtain knowledge on other points in question, many observations were made on the buffer action of leucocytes. In table 12 certain data are presented, selected by virtue of the freedom from red blood corpuscles of these particular leucocyte suspensions. The table shows that when leucocytes are placed in unbuffered solutions of very slight acidity, they absorb H-ions until the pH of the solution is slightly below 7, if their volume is sufficient. (Leucocytes added to a neutral or weakly alkaline unbuffered solution will bring it also to a reaction slightly below pH 7.)

When leucocytes are added to a weakly buffered saline solution such as was used in these experiments, the quantity of H-ions which they absorb depends upon several factors: (1) The total number of H-ions removed from a solution by a given quantity of leucocytes depends upon the nature and quantity of buffer substances in the solution. (2) The dissociation constant of the acid concerned is also a determining factor. A greater quantity of H-ions was absorbed from HCl solutions than from solutions of the tested organic acids of equivalent concentration. Similar quantities of H-ions were absorbed from acetic and butyric acids, which have similar dissociation constants. (3) It was also observed that leucocytes which have already absorbed H-ions have a reduced capacity for further absorption. Haggard and Henderson, and C. L. Evans made similar observations on the buffer action of red blood corpuscles. Their capacity for absorption of H-ions depends upon their previous history (4) It would be expected that a dense suspension of leucocytes would absorb more H-ions from a given quantity of any solution than would a thinner suspension. The experiments showed that to be the case. These observations are in agreement with those made by Gray upon the eggs of trout, from which he drew conclusions for living cells in general.

The data presented in the tables show that leucocytes which have absorbed H-ions have thereby suffered an injury which affects their capacity for phagocytosis. Koltzoff reported that the phagocyting capacity of Carchesium was restored when removed from an acid to a neutral solution. Such was not the case with leucocytes in these experiments.

The cumulative effect of repeated exposures to acid solutions was demonstrated many times. It has already been mentioned in connection with the data presented in table 4. It may also be observed in tables 8, 9, and 10. For example, in table 8, leucocytes whch had been washed once in citric acid solution of pH 4 showed no injury; and leucocytes which were placed in a citric acid solution of pH 4.6 for the final suspension showed no injury if they had not been previously exposed to acid; but when leucocytes which had been previously washed in citric

acid solution of pH 4 were placed in citric acid solution of pH 4.6 for the final suspension, their capacity for phagocytosis was almost completely destroyed.

In agreement with many other investigators who have studied the relative effect of inorganic and organic acids on various kinds of living cells, these experiments show that the organic acids studied (possibly with the exception of citric acid) have a specific toxicity in addition to the toxicity of the free H-ions, as determined by comparison with the toxicity of solutions of HCl, which undergoes practically complete dissociation in the weak dilutions used in these experiments.

It will be recalled that Harvey compared the results of his own investigations and those of several who had preceded him, and that he found little agreement in the order of the various acids relative to their effect on the different living cells studied.

From the data given by Harvey, and that given by Haas and Wyeth in more recent publications, it was possible to tabulate the order of effectiveness on various kinds of living cells of those acids which were used in this study. The comparison, made on the basis of degree of dissociation, is given in table 13. It shows a perfect agreement in the series $\begin{matrix}\text{HCl}\\ \text{citric}\end{matrix} < \text{lactic} < \begin{matrix}\text{acetic}\\ \text{butyric}\end{matrix}$ except that Harvey found citric acid definitely more toxic than HCl. It may be added here that some observations on the relative effect of these acids on red blood corpuscles indicate that this order would not hold for them. Citric acid showed an unquestionably specific toxicity, greater than that of lactic acid.

It has been noted that several investigators have reported that leucocytic activity is stimulated by minute quantities of acids. Hamburger presented data to show that lipoid-soluble substances, including butyric acid, stimulate phagocytosis when present in minute quantities. In the protocol showing the effect of butyric acid on leucocytes (table 10) figures are given for 3 tests in which the leucocytes were treated with weaker solutions of varying strength of butyric acid than the experiment showed to be toxic, yet there was no evidence of a stimulative action in any of them. However, it happened in several experiments

that higher figures for phagocytosis were obtained when the leucocytes had been treated with sub-toxic acid solutions than when they had been treated with neutral solutions. Such examples may be observed in tables 2, 4, 8 and 9. Since, however, such indications of a stimulative action of acids occurred irregularly, and never to a significant degree, it seems more reasonable to regard the mentioned figures as variations due to experimental error.

Practical applications

The experiments show that when phagocytic tests are made *in vitro* it is important to protect the leucocytes against exposure to acid solutions. This is accomplished by the use of a buffered saline solution. It may appear that such a precaution is unnecessary if proper care is taken for the cleanliness of glassware. It is true that the tables show that the leucocytes withstood one washing in an acid solution of pH 4.6 (see tables 8 and 9) without any evidence of an inhibition of phagocytosis. But they showed the injurious effects of much weaker acid solutions when the exposures were repeated. As a matter of fact, before the sensitiveness of leucocytes to acids was taken into consideration it happened rather frequently that the routine quantitative tropin test for the potency of commercial anti-meningococcus serum failed. And here again it may be emphasized that the tropin test has been generally condemned as unreliable.

This is what may happen when all due care is taken for the cleanliness of glassware. The citrate solution used for taking up the pleural exudate may be of an acid reaction. The leucocytes are exposed to a comparatively large quantity of it. Then they are washed in a large quantity of saline solution. If it is unbuffered, and has been allowed to stand with no protection from the air of the laboratory other than the cotton plug, it may have absorbed enough CO_2 from the atmosphere to bring the reaction to as low as pH 5.8. The leucocytes are again exposed to the saline solution in smaller quantity when it is added for making the final suspension. Thus they may be subjected three times to the effects of acid solutions.

The results obtained with the use of acid citrate brings up the discussion of a controversy in the literature in regard to the effect of citrate on phagocytosis. In their early studies on phagocytosis-promoting antibodies Wright and Douglass mixed the blood with sodium citrate to prevent clotting. Their technic has been widely adopted, but Snapper, Ouweleen, Hamburger and Hekma, Radsma, Fenn, and Wolf have all reported that citrate is injurious to leucocytes. On the other hand Stuber and Rütten reported that they could not demonstrate a harmful effect of citrate. Under the conditions of the experiments reported here, a one per cent solution of sodium citrate in the final suspension showed no inhibition of phagocytosis. It seems probable that some of those who reported that citrate was injurious to leucocytes may have been working with an acid product. Ouweleen's statement that the injurious action of sodium citrate was neutralized by serum emphatically suggests that this investigator was working with an acid citrate.

The demonstration of the injurious effect of acid citrate on the phagocytic activity of leucocytes, together with the finding of commercial sodium citrate preparations of an acid reaction, lead to the practical conclusion that when sodium citrate solution is used for preventing coagulation of body fluids containing living cells, it is a matter of importance to ascertain that the solution is not of an acid reaction. On the other hand the leucocytes were apparently unaffected by solutions of slightly alkaline pH values. A solution of the most alkaline citrate preparation encountered in these experiments (pH 8.8) did not harm the leucocytes perceptibly.

Theoretical considerations

The results of these experiments do not indicate definitely whether or not in cases of acidosis the leucocytes may be injured by the acid by-products of the infecting organism. Judging from what is known concerning variations in reaction of the body fluids during life it appears that the leucocytes are protected by the buffer action of the blood against variations in

H-ion concentration which would be injurious. It must be borne in mind, however, that the acids produced by pathogenic bacteria are those which have a specific toxicity for leucocytes. Moreover, the effect on the leucocytes is cumulative. It seems quite possible that leucocytes gathered at the site of an infection and continuously bathed in body fluids containing traces of the acid by-products of the infecting organism may suffer injury which would affect their capacity for phagocytosis.

SUMMARY

Leucocytes absorb H-ions from weakly acid solutions. If the quantity of H-ions absorbed is great enough, the capacity of the leucocytes for phagocytosis is injured.

In addition to the toxicity of the dissociated H-ions, lactic, acetic and butyric acids have a specific toxicity for leucocytes. The order of toxicity of the tested acids is:

When phagocytic tests are carried out *in vitro* it is necessary to protect the leucocytes against the influence of acid solutions. This is accomplished by the use of a buffered saline solution.

Some of the sodium citrate on the market is of an acid reaction and therefore is not suitable for use in the preparation of leucocytic suspensions for the tropin test.

The union of the immune body (bacteriotropin) with the streptococcus was not influenced by such variations of H-ion concentration as were studied in these experiments.

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REFERENCES

Arkin, Aaron: The influence of strychnin, caffein, chloral, antipyrin, cholesteral and lactic acid on phagocytosis. Jour. Infec. Dis., 1913, **13**, 408–424.

Barratt, J. O. Wakelin von: Die Wirkung von Säuren und Basen auf lebende Paramöcien. Z. allg. Physiol., 1904, **4**, 438–484.

Barratt, J. O. Wakelin von: Der Einfluss der Konzentration auf die chemotaxis. Z. allg. Physiol., 1905, **5**, 73–94.

Bechhold, H.: Phagozytosestudien. München. Med. Wohnschr., 1908, **55**, 1777–1778.

Behring, von: Über die Ursache der Immunität von Ratten gegen Milzbrand. Centbl. f. klin. Med., 1888, **9**, 681–690.

Brooks, S. C.: The mechanism of complement action. Jour. Gen. Physiol., 1920, **3**, 185–201.

Bruchman, H.: Von der Chemotaxis der Lycopodium Spermatozoiden. Flora, 1908, **99**, 193–202.

Buller, A. H. Reginald: Contributions to our knowledge of the physioogy of the spermatozoa of ferns. Annals Bot., 1900, **14**, 543–582.

Clark, William Mansfield, and Lubs, Herbert A.: The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact., 1917, **2**, 1–34, 109–136, 191–236.

Cohen, Barnett, and Clark, Wm. Mansfield: The growth of certain bacteria in media of different hydrogen ion concentrations. Jour. Bact., 1919, **4**, 409–427.

Crozier, W. J.: Cell penetration by acids. Jour. Biol. Chem., 1916, **24**, 255–279.

Dragstedt, Lester R.: The effect of Streptococcus hemolyticus infection on the reaction of the blood of rabbits. Jour. Infec. Dis., 1920, **27**, 452–459.

Eggers, H. E.: On the effect of reaction and of certain salts on normal opsonins. Jour. Infec. Dis., 1909, **6**, 662–669.

Evans, C. Lovatt: The regulation of the reaction of the blood. Jour. Physiol., 1921, **55**, 159–192.

Evans, Alice C.: The tropin reactions of antimeningococcus serum. Hyg. Lab. Bull., 1920, **124**, 43–87.

Evans, Alice C.: A buffered physiological salt solution. Jour. Infec. Dis., 1921, **30**, 95-98.

Fenn, Wallace O.: The phagocytosis of solid particles. I. Quartz. Jour. Gen. Physiol., 1921, **3**, 439–464. II. Carbon. Ibid., 465–482.

Fuhner, Hermann, and Neubauer, Ernst: Hämolyse durch Substanzen homologer Reihen. Arch. f. exp. Pathol., 1906, **56**, 333–345.

Gabritchevsky, G.: Sur les propriétés chimiotactiques des leucocytes. Ann. d. l'Inst. Pasteur, 1890, **4**, 346–362.

Garrey, Walter E.: The effects of ions upon the aggregation of flagellated infusoria. Amer. Jour. Physiol., 1900, **3**, 291–315.

Gray, J.: The relation of the animal cell to electrolytes. II. The adsorption of H-ions by living cells. Jour. Physiol., 1920, **54**, 68–78.

Haas, A. R.: The permeability of living cells to acids and alkalies. Jour. Biol. Chem., 1916, **27**, 225–232.

Haggard, Howard W., and Henderson, Yandell: Hemato-respiratory functions. VII. The reversible alterations of the H_2CO_3:$NaHCo_3$ equilibrium in blood and plasma under variations in CO_2 tension and their mechanism. Jour. Biol. Chem., **45**, 189–198.

Hamburger, H. J.: Physikalisch-chemische Untersuchungen über Phagozyten. Bergmann, Wiesbaden, 1912.

Hamburger, H. J., and Hekma, E.: Studien über Phagozytose. III. Beitrage zur Biologie der Phagozyten. Einfluss von Ionen. Biochem. Zeitsch., 1908, **9**, 275–306.

HARVEY, E. N.: Cell permeability for acids. Science, 1914, **39**, 947–949.

HARVEY, E. N.: The permeability of cells for acids. Publications of the Carnegie Institute of Washington, 1915, **8**, 143–156.

HENDERSON, LAWRENCE J.: Equilibrium in solutions of phosphates. Amer. Jour. Physiol., 1906, **15**, 257–271.

HENDERSON, LAWRENCE J.: The theory of neutrality regulation in the animal organism. Amer. Jour. Physiol., 1908, **21**, 427–448.

IRALA, J.: Richerche sulla fagocitosi. Annali d' Igiene, 1920, **30**, 28–34.

JENNINGS, H. S.: Studies on reactions to stimuli in unicellular organisms. IV. Laws of chemotaxis in Paramecium. Ann. Jour. Physiol., 1899, **2**, 355–379.

KAHLENBERG, LOUIS: The action of solutions on the sense of taste. Univ. of Wis. Bull., 1898, **25**, 3–31.

KAHLENBERG, LOUIS, AND TRUE, RODNEY H.: On the toxic action of dissolved salts and their electrolytic dissociation. Bot. Gaz., 1896, **22**, 81–124.

KOLTZOFF, N. K.: Uber die Wirkung von H-ion auf die Phagozytose von Carchesium lachmani. Internationale Zeitsch. f. Physikalische Chemische Biologie, 1914, **1**, 82–107.

LOEB, JAQUES: Physiologische Untersuchungen über Ionenwirkung. Pflüger's Archiv., 1898, **71**, 457–476.

LOEB, JAQUES: Chemische Konstitution und physiologische Wirksamkeit von Alkoholen und Säuren. Biochem. Zeitsch., 1909, **23**, 93–96.

LOEB, JAQUES: Artificial Parthogenesis and Fertilization. University of Chicago Press. Chicago, 1913.

NEUFELD, F.: Ueber die Wirkungsweise und die Wertbestimmung des Genickstarrserums. Med. Klin., Berl. & Wien, 1908, **4**, 1158.

NOGUCHI, HIDEYO: On the influence of the reaction and of desiccation upon opsonins. Jour. Exp. Med., 1907, **9**, 454–463.

OKER-BLUM, MAX: Zum Mechanismus der Bakterienverankerung an das Leucocytenprotoplasma. Zeitsch. Immunität., 1912, **14**, 485–543.

OTSUBO, TORASABURO: On the action of certain salts on phagocytosis and virulence of streptococci. Jour. Inf. Dis., 1921, **28**, 18–26.

OUWELEEN: Invloed van serum op de phagocytose. Dissertation. Gwonigue., 1915. (Quoted from RADSMA.)

PFEFFER, W.: Uber chemotaktische Bewegungen von Bakterien, Flagellaten und Volvocineen, Untersuchungen aus dem Botanische Institut zu Tübingen, 1888, **2**, 582–662.

RADSMA, W.: Contribution a la biologie de la phagocytose de l'homme. Archives Neerlandaises de physiologie de l'homme et des animaux, 1918, **2**, 301–318.

RICHARDS, THEODORE WILLIAM: The relation of the taste of acids to their degree of dissociation. Amer. Chem. Jour., 1898, **20**, 121–126.

SAWTCHENKO, I. G., AND ARISTOWSKY, V. M.: Sur l'importance de la reaction du milieu pour la phagocytose. Arch. Sci. Biol., 1912, **17**, 128–138.

SCHÜTZE, H.: Studies in spontaneous phagocytosis. Jour. Hyg., 1914, **14**, 201–214.

SCHWYZER, FRITZ: Die Oberflächenspannung der Leucocyten und deren Beeinflussung. Biochem. Zeitsch., 1914, **60**, 306–309.

SCHWYZER, FRITZ: Beobachtungen an Leucocyten bei Variationen der Ionenkonzentration. Biochem. Zeitsch., 1914, **60**, 447–453.

SHIBATA, K.: Studien über die Chemotaxis der Isoëtes-Spermatozoiden. Jahrb. wiss. Bot., 1905, **41**, 561–610.

SNAPPER, I.: Phagocytose bij den mensch. Nederlandsch Tijdschrift voor Geneeskunde, 1914, **50**, 2000–2010.

Standard methods of water analysis. Amer. Pub. Health Assoc., 4th edition, 1920, p. 4.

STUBER, B., AND RÜTTEN, F.: Ueber eine einfache Methode zur Bestimmung des phagozytären Index und dessen klinische Bedeutung Münch. Med. Wochenschr., 1913, **60**, 1585–1587.

VAN SLYKE, DONALD D., AND CULLEN, G. E.: Studies of acidosis. I. The bicarbonate concentration of blood plasma; its significance and its determination as a measure of acidosis. Jour. Biol. Chem., 1917, **30**, 289–346.

VAN SLYKE, DONALD D.: The normal and abnormal variations in the acid-base balance of the blood. Jour. Biol. Chem., 1921, **48**, 153–176.

WELLS, H. GIDEON: Chemical Pathology. 3rd edition. Saunders, Philadelphia. 1918.

WINSLOW, C. E. A., AND LOCHRIDGE, E. E.: The toxic effect of certain acids upon typhoid and colon bacilli in relation to their degree of dissociation. Jour. Infec. Dis., 1906, **3**, 547–571.

WOLF, ELIZABETH PAULINE: Experimental studies on inflammation. Jour. Exp. Med., 1921, **34**, 375–396.

WOLF, CHARLES GEORGE LEWIS, AND HARRIS, JOHN EDMUND GUY: Contributions to the biochemistry of pathogenic anaerobes. III. The effect of acids on the growth of Bacillus welchii (B. perfringens) and Bacillus sporogenes (Metchnikoff) Biochem. Jour., 1917, **11**, 213–245.

WRIGHT, ALMOTH E.: Conditions which govern the growth of the bacillus of "gas gangrene" in artificial culture media, in the blood fluids in vitro and in the dead and living organism. Lancet, 1917, **1**, 1–9.

WRIGHT, A. E., AND DOUGLAS, STEWART R.: Investigation of the rôle of the blood fluids in connection with phagocytosis. Proc. Roy. Soc. (Lond.), 1903, **72**, 357–370.

WYETH, FRANK JOHN SADLER: The effect of acids on the growth of B. coli. Biochem. Jour., 1918, **12**, 382–401.

A SEROLOGICAL STUDY OF THE GONOCOCCUS GROUP[1]

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The primary purpose of this investigation has been to determine whether gonococci may be distributed among a number of fixed immunological types or whether, on the other hand, the strains exhibiting dissimilar serological characteristics may more logically be considered as more or less labile variants from a common basal type.

In 1907, one of us (1) published the results of a similar study of this group and arrived at the conclusion that based on agglutination and agglutinin absorption tests, the gonococcus group embraces a heterogeneous collection of types. Among the ten strains studied, three types were recognized to which, however, only six of these strains could be referred. It was realized at the time that the serological relationships within the group were complex and that there existed "all manner of intermediate forms and variations." About this time it was shown by Teague and Torrey (2) that complement fixation tests with these cultures added confirmation to our conclusions in regard to the absence of serological uniformity in this group and indicated the desirability of using several different gonococcus strains in preparing antigens for use in clinical diagnosis.

In view of these findings Dr. H. J. Schwartz and Dr. Archibald McNeil in connection with their investigation of the clinical application of complement fixation tests to the diagnosis of gonococcus infections (3) utilized such of our strains as were then

[1] This investigation was made possible by a grant from the U. S. Interdepartmental Social Hygiene Board, Washington, D. C.

available. Of the twelve strains which they received from us, six (A, B, C, G, H, J) had been subjected to serological analysis and six had been subsequently added to the collection without special study. Nine of these strains with one other, S, the origin of which is not known, have been carried in various laboratories to the present time under the designation of "Torrey strains," and have been used quite extensively in the preparation of gonococcic antigen for complement fixation work. These details are published here because of the rather widespread impression that these ten strains had been found to represent ten different gonococcus types. This, however, has never been claimed to be the case as reference to the original articles will show.

That the determination of the serological unity or diversity of strains embraced within a bacterial species of pathogenic propensities is of more than academic interest, is evidenced by the large amount of labor which has been devoted to the study of "types" within the pneumococcus, meningococcus, typhoid, tetanus, influenza and other bacterial groups. The results of some of this work has had a very practical bearing, not only on specific therapy, but also on the epidemiological aspects of these infectious diseases. Up to the present time only a comparatively few serological studies of the gonococcus group have appeared in the literature and to these reference will be made later on.

As regards this present study the particular objectives held in view have been the selection of strains most suitable for complement fixation antigens, the determination of the most representative strains and those with marked antibody stimulating propensities for use in stock vaccines, and also the possible application of these selected strains to the production of curative sera. In addition, of course, our attention was directed to certain epidemiological considerations such as the relationship of strains causing vulvovaginitis in children to those giving rise to gonorrhea in adults.

METHODS

Media. A description will be given in this article only of the media used directly in connection with this serological study, while the cultural procedures found especially efficacious in the isolation of the gonococcus and in the maintenance of stock strains will be dealt with in another communication.

The following ascitic agar medium, which for convenience we have designated as 12 (a), has been used exclusively in obtaining growth for the agglutination experiments. Uniformity in constitution and reaction has been maintained as far as possible. This solid medium yields an unusually heavy growth for gonococcus and generally one which emulsifies readily. Large slants in tubes 1 by 8 inches in size have been used.

Fifteen hundred grams of fresh, fat-free, finely chopped veal are mixed with $2\frac{1}{2}$ liters of distilled water and placed in a covered pot over a low flame. The temperature is gradually raised to the boiling point and the medium allowed to simmer for twenty minutes, with occasional stirring. It is then strained (with pressure) through canton flannel, cooled and the fat removed. It is next placed in a double boiler over a saturated brine bath, the temperature raised to about 60°C. and 1 per cent peptone (Difco), 2 per cent fresh urine, 2 per cent glycerine, 0.5 per cent NaCl and 2 per cent flaked agar are added. This mixture is allowed to boil for forty-five minutes. The reaction is then adjusted to pH 6.9 (using a 10 per cent solution of sodium carbonate), and the boiling is continued for thirty minutes. The loss from evaporation up to $2\frac{1}{2}$ liters is replaced with distilled water. It is next filtered through canton flannel and about 15 cc. are placed in each of the large tubes noted above. Sterilization is effected by heating in the autoclave at 12 pounds pressure for 10 minutes. In preparing the slants ascitic fluid, free from bile, is added in the proportion of one part to four or five of the above medium. The final reaction is generally close to pH 7.2. The slants should not be prepared longer than the day before they are to be used.

The collection of gonococcus strains has been maintained on a

semi-solid vitamine medium prepared, with a slight modification, according to the formula given by Huntoon (4). The marked advantages of this medium in carrying a large series of gonococcus cultures will be discussed in another place.

The medium used in connection with the preparation of antigen for the complement fixation experiments is described in the section dealing with that work.

Animal immunizations. One of the first questions considered in connection with this study was the type of gonococcus antigen which possessed the best agglutinogenic properties. Comparative tests were first made with living and (heat) killed gonococci. The results of a test of this character are given in table 1. The two rabbits received exactly the same dosage of antigen, given at the same time intervals; the only difference lay in the fact that to one (222) living antigen and to the other (223) antigen heated at 55°C. for fifteen minutes were given.

Perry and Kolmer (5), in a comparative study of the immunizing properties of *B. typhosus* vaccine treated in various ways, reported that, as far as their agglutinogenic potentialities were concerned, they might be ranked in the following order: living and autolyzed; mercophen and tricresol killed; heat killed; and lastly, alcohol killed sensitized sediment. We have conducted some similar experiments using gonococcus strain 33 and killing the antigen through the use of (*a*) tricresol, 0.25 per cent, (*b*) acetone, (*c*) chloroform, (*d*) heated at 55°C., and (*e*) at 65°C. for fifteen minutes. Rabbits were immunized with equal amounts of each of these variously treated antigens and, after six inoculations, agglutination tests were carried out with the homologous strain. Much better results were obtained with the heat-killed and also with the chloroform treated antigens than with those treated in the other ways specified. It was decided, then, as a routine procedure to use antigens exposed to 45°–50°C. for fifteen minutes, as this degree of heat was found sufficient to kill the gonococci and would be likely to cause no change in its antigenic properties.

Some of the rabbits used in the production of the immune sera were injected at weekly intervals and others were given more intensive treatment by inoculations on three consecutive days a

intervals of four to seven days. The latter procedure yielded much the better results, both as regards the titer of the serum produced and the time consumed. All of the injections were given intravenously. The amount of the dosage of standard

TABLE 1

Comparative agglutination test with serums of rabbits immunized with living and heat (55°C.) killed gonococcus antigen. Rabbit 222 received living strain 41. Rabbit 223 received killed strain 41. The experiment was conducted at 55°C. and the results read after twenty-four hours.

STRAINS	SERUM NUMBERS	NORMAL SERUMS	AFTER 3 INOCULATIONS	AFTER 9 INOCULATIONS
1	222	<25	50	100
	223	<25	100	100
5	222	<25	50	250
	223	<25	100	250
9	222	50	1500	3000
	223	50	1500	3000
18	222	<25	100	1500
	223	<25	250	1000
20	222	<25	100	2000
	223	<25	500	1500
25	222	<25	25	100
	223	<25	25	100
41 Homologous	222	<25	250	2000
	223	<25	750	1500

This experiment indicated that there was little choice between living and heat-killed agglutinogen, although after nine inoculations a slightly higher titer serum was obtained with the living culture.

suspensions was regulated entirely by the condition of the animal. The sera were preserved, for the most part, with 0.1 per cent phenol.

Preparation of antigens for agglutinations. In preparing the bacterial suspensions for agglutination experiments, a standard procedure was carefully followed. The gonococcus strains were grown on the ascitic agar (12a) which has been described. It

was found advantageous to seed these slants from twenty-four hour growths on the same medium. They were incubated at 36° to 37°C. for twenty-four to forty-eight hours. Before emulsifying the growth, the water of condensation and hysteresis was removed by washing out with normal saline solution. This saline solution was prepared with chemically pure NaCl, 0.85 per cent in distilled water. In some of the experiments 0.5 per cent formalin was added to the salt solution, both because this agent preserved the gonococci perfectly for an indefinite period and also in that it tended to check spontaneous clumping. Although Sands (6) found in connection with typhoid agglutination experiments that especially satisfactory results were obtained if the bacilli were emulsified in saline containing 1 to 2 per cent formalin and Hooker (7) also advocates formalinized saline for typhoid agglutination, it has been our experience with the gonococcus that even 0.5 per cent of formalin causes a considerable decrease in sensitiveness to the action of agglutinin, which is much more marked in connection with some strains than in that of others. Such being the case formalinized emulsions were discarded for freshly prepared living suspensions. Gordon (8) and a number of other investigators of serological relationships within the meningococcus group have used emulsions heated at 65°C. for one-half hour, to destroy the autolysin, with 0.5 per cent phenol added as a preservative. It has been our experience that this degree of heat is insufficient to affect greatly the gonococcus autolysin. McClintock and Clark (8) have also reported that temperatures of less than 70°C., with or without the addition of tricresol up to 0.4 per cent, do not prevent the disintegration of suspensions of gonococci in salt solution. With certain recently isolated strains we have found that heating for one-half hour at 65°C. increases markedly the viscocity of the emulsion. Sands (6) noted that heating the typhoid bacillus at 60°C. generally increased their susceptibility to specific agglutinins. We have not noted, however, any enhanced sensitiveness to agglutinins on the part of gonococci after treatment with heat. As there is a good deal of risk of affecting the antigenic properties by heating at 70°C. or higher, and other methods for

destroying the autolysin seemed still more objectionable, we have found it necessary to use living untreated emulsions.

Standardization of gonococcal suspensions. In order that agglutination tests within bacterial groups may have definite comparative value, it is necessary that the density of the culture suspensions be carefully standardized. To obtain such uniformity in the dosage of antigen we adopted the following procedure: After washing and emulsifying the twenty-four to forty-eight-hour growth in the saline solution, as described in the preceding section, the suspension of gonococci was filtered through washed absorbent cotten into a test tube 18 mm. in diameter. Saline was then added until the density of suspension was such that the edge of a wax pencil mark on the back of the tube was just definitely discernible by the observer with his back to a good light. This suspension gave a count of approximately three billion organisms per cubic centimeter, which, when mixed with equal parts of the serum dilution, was reduced to about 1½ billions. In setting up the agglutination tests a rather narrow (8 mm. inside diameter) serological tube was used, and to 0.5 cc. of the serum dilution, 0.5 cc. of the bacterial suspension was added.

Incubation and reading of the tests.—Incubation of the tests was conducted at 50° to 55°C. through the use of a water bath. This temperature is preferable to 37°C. in that it accelerates the reaction and also, in some degree, tends to inhibit spontaneous clumping. During the early part of the experimental work the tubes were kept at this temperature for eighteen to twenty-four hours and then read, but later, after an incubation of two hours, the tubes were placed over night in the ice-chest; there was no appreciable difference in the results. Readings were made at the one-, two- and eighteen- to twenty-four-hour periods. The last period reading was adopted as the final result but the earlier observations often yielded important information, especially when spontaneous precipitation was a confusing factor.

This tendency to spontaneous clumping on the part of certain strains of gonococci was a troublesome feature in the agglutination work and no way was found to eliminate it entirely with

certain strains. This tendency was not confined to certain old stock strains but was noted, in a few instances, as a characteristic of recently isolated ones. The saline control tube gave, of course, a clue to the amount of non-specific clumping and precipitation, but the best method for discounting this factor lay in a careful inspection of the character of the sediment in tubes showing apparent agglutination. If the sedimentation was non-specific, a gentle shaking of the tube broke up the clumps and brought about an even suspension of the cocci. With true agglutination, on the other hand, the clumps are generally large, firm and compact, and are not disintegrated by a fairly sharp shake of the tube; as a rule, the more complete the agglutination, the larger and more firmly united are the clumps. It should be added that spontaneous agglutination was an annoying factor in connection with only a small minority of the strains. At times it was found advantageous, after making a reading at the twenty-four hour period, to shake up the tubes and reincubate them for a few hours. Following this procedure the end point was sometimes more sharply indicated.

The end point selected, as marking the agglutination titer, was the last dilution tube showing complete or almost complete clumping and sedimentation of the organisms; a tube given this reading showed either a clear condition or no more than a very faint clouding of the supernatant fluid (the latter reaction was read as + + +). Reactions of less degree than this are deceptive in character and do not serve as a trustworthy guide to the true titer of a serum.

SOURCE OF THE GONOCOCCUS STRAINS

This collection of gonococcus strains is quite unique in that the infection of a considerable number of cases, from which successful isolations were made, was known to have occurred in widely separated foreign countries and also in different parts of this country. Our strains, perhaps, are thus more nearly representative of the gonococcus group than any collection which has been studied serologically heretofore. The origin of these cultures, where it is of interest, will be noted in subsequent sections.

The ten so-called "Torrey strains" are designated by the numbers 24, 29, 30 to 37. These strains have been under cultivation for about fourteen years; other strains of about the same age are numbers 21 and 23.

As regards the type of infection from which these strains were isolated, forty-seven were from cases of acute and chronic urethritis of males, four from genito-urinary infections of females, four from joint cases, two from septicaemias, one from an eye infection and thirteen from vulvovaginitis infections of children.[2]

AGGLUTINATION

With a number of bacterial species agglutination tests alone, without absorptions, have been found to serve as a quite trustworthy guide to their distribution among serologically distinct types. Such tests, for instance, are generally sufficient for the typing of pneumococci and also, according to some investigators, even the meningococci. In reference to the latter, Butterfield and Neill (9) have reported that 90 per cent of their meningococci strains could be typed by straight agglutinations alone, using Gordon's type serums prepared with Gordon's four type cultures. With the gonococcus group, however, straight agglutination tests have yielded almost no clue to specific serological relationships.

In table 2 are reported some agglutination results with our first forty-seven strains. The figures in the first five columns are particularly comparable in that the rabbits immunized respectively against strains 33, 1, 5, 8 and 13 had received ex-

[2] For the opportunity and for assistance in culturing these infections in children we are greatly indebted to Dr. B. Wallace Hamilton who extended to us the facilities of his service in the Vanderbilt Clinic. For the cultures from women, the "Torrey strains" and several others, our thanks are due to Miss M. A. Wilson, Bureau of Laboratories, New York City Health Department. We also gratefully acknowledge the kindness of Parke, Davis and Company in supplying us with some of the "Torrey strains" and also several other cultures; and of H. K. Mulford Company for a duplicate set of some of these strains. All of the foreign strains were isolated from cases at the U. S. Public Health Service Clinic in this city and for this opportunity we are indebted to the director, Dr. Clifton, and to Dr. Delzell for much helpful assistance. Some other strains, isolated at this clinic, were kindly given to us by the late Dr. E. Finch.

TABLE 2

Agglutination reactions of gonococcus cultures with gonococcus sera. Rabbits 218, 219, 220, 221 and 861 had received identical treatment as regards the number (12) and dates of inoculations and the dosage. The homologous titers are in heavier type*

STRAINS	RABBIT SERA IMMUNE TO GONOCOCCUS STRAINS							
	33 (rabbit 218)	1 (rabbit 219)	5 (rabbit 220)	8 (rabbit 221)	13 (rabbit 861)	29 (rabbit 217)	41 (rabbit 223)	7 (rabbit 230)
1	<25	**100**	50	0	0	100	<50	<100
2	0	100	<25	0	0	0	<50	<100
3	0	2000	50	100	250	250	250	4000
4	50	<25	<25	25	250	0	0	1000
5	50	100	**250**	25	50	<50	100	100
6	<25	1000	50	100	750	250	500	1000
7	50	250	100	0	25	500	250	**3000**
8	25	250	250	**250**	250	<25	250	1000
9	3000	3000	250	1000	1000	1500	2000	
10	1000	250	100	250	50	500	500	2000
11	2000	700	750	750	2000	250	1500	1000
12	100	50	50	25	250	<50	50	100
13	<25	50	25	0	**25**	<50	<50	<100
14	750	100	25	100	100	25	1000	100
15	1000	1000	<25	750	250	500	500	2000
16	100	250	25	<25	100	0	100	<100
17	250	100	250	25	100	25	100	<100
18	100	100	500	750	100	25	50	100
19	2000	700	100	250	2000	1500	1500	<100
20	750	50	<25	50	100	100	100	4000
21	250	<25	750	50	100	500	100	<100
22	0	50	25	0	50	<25	<50	<100
23	1000	250	750	750	5000	1500	1500	4000
24	0	50	25	250	0	1500	250	2000
25	100	250	250	500	100	0	<50	<100
26	25	1000	25	25	0	50	<50	100
27	<25	50	50	250	25	0	50	<100
28	0	0	<25	<25	0	0	<50	<100
29	750	250	25	<25	1000	**500**	1500	3000
30	750	1000	1000	250	750	500	2000	4000
31	750	25	250	<25	250	1000	1500	4000
32	2000	3000	1000	500	750	3000	2000	4000
33	**1000**	1000	750	250	750	1500	1000	4000
34	1000	700	1000	250	250	1000	3000	4000
35	250	700	2500	250	100	1500	1000	4000

* A small dosage was used in the immunization of all these five rabbits.

TABLE 2—*Continued*

STRAINS	RABBIT SERA IMMUNE TO GONOCOCCUS STRAINS							
	33 (rabbit 218)	1 (rabbit 219)	5 (rabbit 220)	8 (rabbit 221)	13 (rabbit 861)	29 (rabbit 217)	41 (rabbit 223)	7 (rabbit 230)
36	<25	1000	250	250	750	1000	2000	3000
37	1000	1000	1000	1000	2000	<50	500	2000
38	500	250	100	500	750	50	250	1500
39	50	<25	250	50	25	<50	250	<100
40	250	700	100	100	750	0	50	1500
41	25	500	50	100	0	<50	**250**	500
42	750	500	750	750	750	<50	1500	4000
43	50	100	<25	100	<25	0	50	<100
44	<25	100	250	50	0	100	50	1000
45	0	250	25	500	500	<50	100	1500
46	<25	100	25	100	<25	0	50	<100
47	1000	250	50	100	250	<50	500	3000

actly similar treatment as regards dosage, inoculation intervals and time of bleeding.

The inconsistencies, as far as possible typing is concerned, revealed by straight agglutination tests, may be illustrated by the reactions of strain 33 and its serum (218). This serum agglutinated strains 1, 5, 8 and 13 only at a dilution of 1:25 or less and yet a serum produced to each one of these strains agglutinated strain 33 in dilutions as high or much higher than their respective homologous titers (table 2). An inspection of this table also shows that, with the exception of strain 8 with strain 7 serum, these strains agglutinated poorly with all of the eight sera and their failure to react with strain 33 serum may be as reasonably ascribed to their poor agglutinating qualities as to the absence of specific or group agglutinins to which they are sensitive. Straight agglutination tests, then, are not of any definite value in indicating possible type relationships between gonococcus strains. Warren (11) in a recent study of the agglutination reactions of twenty-three gonococcus strains came to a similar conclusion, but for the reason that all the strains agglutinated about equally well with the three or four sera tested.

Gonococci are reputed, as a class, to be more sensitive to the action of agglutinins than are the meningococci. Although this

may be in a measure true in that a much higher titer may be obtained through inoculations with certain gonococcus strains, especially those cultivated for some time, than is possible with meningococci and their respective anti-sera, yet poorly agglutinating gonococcus strains have been quite frequently encountered, especially when tested soon after isolation. Among sixty-three recently isolated strains, twenty-six were found to be relatively inagglutinable with the test sera. Of course failure to react may have been due in some instances to absence of group agglutinins to which the strains were sensitive, but with this reservation the fact that 42 per cent of these gonococcus strains were apparently relatively inagglutinable corresponds closely with the 40 per cent of similarly inagglutinable meningococcic strains reported by Elser and Huntoon (10) as occurring in their series of cultures. Some gonococcus strains, relatively inagglutinable soon after isolation with the sera employed, became later much more sensitive to the action of agglutinins, whereas other strains, such for example as 2, 5, 13, 16, 22, 28 and 46, did not acquire under cultivation any increased susceptibility to the action of agglutinins. We have, however, encountered no instance of a strain becoming inagglutinable under cultivation as was reported by Elser and Huntoon to have occurred in reference to certain of their meningococcic strains. Attempts were made to increase the sensitivity of some of our inagglutinable strains, but neither growing them in glucose broth for several generations—a method applied successfuly with meningococci by Elser and Huntoon—nor by a period of daily transplants, was their agglutinability enhanced.

Elser and Huntoon (10) observed that, although the most agglutinable of their strains of meningococci were likely to produce the more potent agglutinating sera, there was no definite relationship between agglutinability and agglutinogenic properties. We have found this to be true also for gonococci and, as is shown in table 2, sera produced with certain relatively inagglutinable strains (1, 5, and 13), although giving a low titer for themselves, may agglutinate other strains in high dilutions. These strains may also be capable of absorbing well.

It has been the common experience of those who have studied extensively the agglutination of the meningococcus and also of the gonococcus that the agglutinability of a given strain may exhibit a considerable degree of variation when tested from time to time with the same serum. These variations are probably

TABLE 3

Agglutination of recently isolated gonococcus strains and relatively inagglutinable stock strains with a serum immune to strains 15 and 41. Titer for homologous strain 15 = 30,000. Titer for homologous strain 41 = 2000.

RECENTLY ISOLATED STRAINS							RELATIVELY INAGGLUTINABLE STOCK STRAINS					
Strains	Generation number	Serum dilutions					Strains	Serum dilutions				
		100	200	500	1000	2000		100	200	500	1000	2000
J	3	4	4*	3	—	—	1	4	4	2	—	—
01	4	4	4	4	2	—	2	4	4	3	—	—
02	4	4	3	2	—	—	5	4	3	—	—	—
03	4	4	2	1	—	—	12	2	2	—	—	—
04	3	4	4	4	1	—	16	4	4	—	—	—
05	3	4	4	4	4	—	20	4	4	4	4	3
06	4	4	4	3	2	—	21	4	4	4	3	2
07	4	4	4	4	4	2	22	4	2	—	—	—
91334	4	4	4	—	—	—	25	4	2	—	—	—
08	4	4	3	—	—	—	39	4	4	4	3	2
09	4	4	4	3	2	—	45	4	4	3	3	—
010	5	4	2	—	—	—	53	4	3	—	—	—
011	5	4	4	4	4	2	55	4	2	—	—	—
012	4	4	4	4	4	4	56	4	—	—	—	—
013	3	4	2	—	—	—	57	3	—	—	—	—
014	3	3	1	—	—	—	59	4	3	—	—	—
015	3	4	3	1	—	—	62	4	3	—	—	—
016	3	4	4	4	4	3	77	4	4	—	—	—

* 4 indicates complete agglutination; 3, nearly complete agglutination.

referable to slight differences in the physical characteristics of the growth due to slight changes in the condition of the medium or the environment, especially as regards moisture. In experiments of a comparative nature this tendency of the gonococcus to vary in agglutinability must be constantly borne in mind and controlled with great care.

With a single exception none of the monovalent agglutinating sera gave promise of being of much value for the identification of the gonococcus. The serum giving the best results was one of a high titer immune to strain 15 (table 5) which agglutinated sixty-seven of the seventy-four strains (or about 90 per cent) in a dilution of 1:250 or higher. Several polyvalent sera prepared through immunization with two or more selected strains have been fairly effective, but none covered our entire collection of strains. Perhaps the best combination of any two of our strains is that of 15 and 41. The results with a high titer serum produced with this combination is shown in table 3. In this table are given the titers obtained with a series of very recently isolated strains and also that of some of the relatively inagglutinable strains which had been under cultivation for many months. It may be said in general that a positive agglutination with a gonococcic serum at a dilution of at least 1:250 definitely identifies the culture under consideration as gonococcus but that a negative result has no certain significance as some undoubted strains of gonococci are practically inagglutinable.

ABSORPTIONS

The greater part of the time occupied by this study has been devoted to agglutinin absorption tests to determine whether our series of gonococcus strains may be distributed through this method of classification among a number of clear-cut, immunological types or, if not, in what way the various strains are related. In conducting these tests our effort has been to maintain the conditions as nearly uniform as possible.[3]

Methods

In order to obtain sufficient growth for these absorptions, the strains were seeded on large potato tube slants of ascitic agar, prepared as heretofore described and with a reaction close to pH 7.2. Plates were used at first but proved inferior to the

[3] In much of this absorption work and also in the complement fixation tests we have received valuable assistance from Luther B. Conklin.

slants. These slants had each a surface area of approximately 2500 sq. mm. It was found advantageous to make plantings from twenty-four-hour growths on the same medium. Generally three tubes were seeded with a single strain for an absorption. After twenty-four to forty-eight hours incubation the growths were washed with the saline solution and then suspended in a small amount of the same. The suspension of gonococci was then placed in a graduated centrifuge tube and whirled until 0.2 cc. of rather loosely packed organisms had been deposited in the tip of the tube. In thus measuring the absorbing dose of culture we are following the method used by Valentine and Cooper (12) in their serological study of the *B. influenzae.* This method, as applied to gonococci, is not strictly accurate for comparative experiments owing to a number of uncontrollable factors, such as the amount of autolysis, but is perhaps as satisfactory as any which may be utilized. Generally not all of the growth from the three slants was required for the absorbing dose and the excess was used in determining the agglutination titer of the unabsorbed serum for the particular strain. With every serum an absorption was carried out with this dosage of a meningococcus strain in order to control the factor of non-specific absorption. This test of specificity was of much value as evidence of non-specific absorption occasionally appeared in connection with low-titer sera. The serum to be absorbed was diluted to the proper degree (this should be approximately the lowest dilution at which the homologous strain in the dosage used will completely absorb its agglutinins) and the centrifuge tube containing the sedimented bacteria was filled to the 5-cc. mark with it. An even suspension was then made of the growth in the diluted serum, and the tube placed in the water bath at 45° to 50°C. for two hours, it being shaken at intervals of about fifteen minutes. Unabsorbed serum, diluted to the absorption titer, was exposed to this temperature for the same time period. Both the absorbed and the unabsorbed serum lots were then centrifuged until the former tubes were clarified fully or to a sufficient degree for the purposes of the test. Centrifugation of the unabsorbed serum was considered advisable in view of the finding of Elser and Huntoon

that the vibration of the machine might cause a lowering of its titer. Suitable graded dilutions of the unabsorbed and absorbed lots of the serum were then prepared and agglutination tests set up by which the following titers were determined: that of the absorbing strains with the unabsorbed serum; the absorbing strains with their respective lots of absorbed serum; the homologous strain with each lot of absorbed serum and also with the unabsorbed serum. The last two determinations with the homologous strain are, of course, the all important ones in tracing specific relationships, but the others give much additional information or serve as important controls. The absorption tests were incubated and readings made as described for the straight agglutination experiments.

In a number of recent studies of type determinations among bacterial groups through absorption methods, an absorbing period of three to six hours at 50° to 55°C. has been employed. Hermanies (13), in a study of this character of the gonococcus, incubated his absorptions at 55°C. for four to six hours, left the tubes overnight in the ice-chest, and carried out the tests the next day. As our absorptions and tests were always carried through on the same day, we have employed a two hour absorption period, and believe that the agglutinin is fully bound within that period. This opinion seems substantiated by the comparative test reported in table 4. Some differences in the degree of absorption may be noted, especially marked as regards strain 25, but these discrepancies should probably be referred to the apparently inevitable variations associated with agglutination of gonococci rather than specifically to the absorption time factor. It should be noted that these two tests were not carried out on the same day, but this rather adds to than detracts from their comparative interest.

In the attempts to distribute our gonococcus cultures among distinct serological types, the first fifty strains were used for the most part, although with three sera the tests were extended to include all but a few of the total seventy-seven strains. In all these experiments the absorption of specific or homologous agglutinins was alone determined and the question of relationships

through so-called minor or group agglutinins was not approached. By way of illustration a complete report is made in table 5 of the results with one of the test sera on all the strains then available. It should be noted that the percentages of homologous agglutinin absorptions are stated in several instances in approximate rather

TABLE 4

Table to show the comparative effect of absorptions at the following time periods: A. Absorbed at 55°C. for two hours. B. Absorbed at 55°C. for six hours and left in ice box over-night. Serum 104, immune to culture 15. Absorbed at dilution of 1:250*

CULTURES	TITER OF ABSORBING CULTURE WITH UNABSORBED SERUM	TITER OF ABSORBING CULTURE WITH ABSORBED SERA	TITER OF HOMOLOGOUS CULTURE WITH ABSORBED SERUM	TITER OF ABSORBING CULTURE WITH UNABSORBED SERA	TITER OF HOMOLOGOUS CULTURE WITH ABSORBED SERA	PERCENTAGE OF ABSORPTION	
	A			B		A	B
1	500	<500	1000	<500	500	87.5	93.8
7	8000	<500	4000	<500	4000	50.0	50.0
11	16000	<500	1000	<500	<500	87.5	100.0
15 Homologous	8000	<500	500	<500	<500	100.0	100.0
18	4000	<500	2000	<500	2000	75.0	75.0
19	8000	<500	500	<500	500	93.8	93.8
25	500	<500	500	<500	4000	93.8	50.0
30	8000	<500	500	<500	1000	100.0	87.5
34	8000	<500	2000	<500	2000	75.0	75.0
41	500	<500	8000	<500	8000	0.0	0.0
49	2000	<500	8000	<500	8000	0.0	0.0
Meningococcus	<250	<500	8000	<500	8000	0.0	0.0

* Titer homologous strain 15 with serum 104 = 8000.

than exact figures; repeated tests would show too many variations to justify the presentation of very exact figures in reference to a single experiment. These percentages, however, give a sufficiently accurate idea of the serological relationships of the several strains to the homologous culture.

As has already been stated this serum 104 immune to strain 15 gave positive agglutinations with a greater number of our strains than any other tested as extensively. An examination of

TABLE 5

Strain 15 serum (104) absorbed with each of gonococcus strains 1 to 77 and with meningococcus, and tested as shown below. Serum absorbed at 1:250 dilution

STRAIN	TITER UNABSORBED SERUM	TITER ABSORBING STRAIN WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
1	500	<500	8000	1000	87
2	1000	<500	8000	8000	0
3	4000	<500	8000	2000	75
4	1000	<500	8000	4000	50
5	<250	<500	8000	4000	50
6	8000	<500	8000	2000	75
7	500	<500	8000	4000	50
8	1000	<500	8000	1000	87
10	8000	<500	8000	2000	75
11	4000	<500	8000	<500	100
12	500	<500	8000	1000	87
13	1000	<500	8000	8000	0
14	1000	<500	8000	2000	75
15*	8000	<500	8000	<500	100
16	500	<500	8000	8000	0
17	1000	<500	8000	8000	0
18	4000	<500	8000	2000	75
19	1000	<500	8000	500	93
20	500	<500	6000	6000	0
21	500	<500	6000	4000	33
22	<250	<500	6000	6000	0
23	8000	<500	6000	1000	84
24	8000	<500	6000	<500	100
25	500	<500	8000	500	93
26	1000	<500	6000	1000	84
27	1000	<500	6000	4000	33
28	2000	<500	6000	2000	66
29	8000	<500	6000	500	92
30	8000	<500	8000	<500	100
31	8000	<500	6000	1000	84
32	8000	<500	6000	1000	84
33	6000	<500	6000	1000	84
34	8000	<500	8000	2000	75
35	8000	<500	8000	500	100
36	250	<500	6000	1000	84
37	6000	<500	6000	1000	84
38	4000	<500	8000	4000	50
39	500	<500	6000	2000	66

* Homologous.

TABLE 5—*Continued*

STRAIN	TITER UNABSORBED SERUM	TITER ABSORBING STRAIN WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
40	2000	<500	6000	2000	66
41	500	<500	8000	8000	0
42	4000	<500	6000	500	92
43	<250	<500	6000	<500	100
44	2000	<500	6000	500	92
45	250	<500	6000	2000	66
46	<250	<500	6000	2000	66
47	2000	<500	6000	4000	33
49	2000	<500	8000	8000	0
50	4000	<500	6000	1000	84
51	2000	<500	6000	500	92
52	250	<500	6000	500	100
53	250	<500	6000	1000	84
54	2000	<500	6000	2000	66
55	500	<500	6000	4000	33
56	<250	<500	6000	4000	33
57	250	<500	6000	2000	66
58	500	<500	6000	6000	0
59	250	<500	6000	2000	66
60	4000	<500	6000	1000	84
61	8000	<500	8000	500	92
62	500	<500	6000	4000	33
63	8000	<500	6000	500	92
64	8000	<500	8000	2000	75
65	1000	<500	8000	4000	50
66	16000	<500	8000	1000	87
67	2000	<500	8000	8000	0
69	6000	<500	8000	2000	75
70	4000	<500	6000	500	92
71	8000	<500	8000	500	93
72	<250	<500	6000	6000	0
73	2000	<500	8000	500	93
74	8000	<500	8000	6000	25
75	8000	<500	8000	8000	0
76	2000	<500	8000	8000	0
77	250	<500	8000	6000	0
Meningococcus	<250	<500	8000	8000	0

table 5, however, shows that a high agglutinating titer with the serum does not necessarily mean a specific relationship to the serum strain; for example strains 43, 52 and 53 gave a titer of

only 1:250 or less and yet these cultures were able to absorb the homologous agglutinins strongly, on the other hand strains 74 and 75 agglutinated to the full titer of the serum and yet absorbed little or none of the homologous agglutinins. Many other examples of a similar discrepancy between agglutinating and absorbing capacities may be found in this table and, in fact, were encountered frequently in the course of this investigation. These facts indicate that straight agglutination tests with gonococci are not as trustworthy a guide to specific relationship as they appear to be for the meningococcus and certain other bacterial groups.

Absorption tests of the same type as that illustrated by table 5 were carried out with nine other sera. The serum strains used were fairly representative of our whole collection and were derived from widely separated geographical localities. It is neither feasible nor desirable to present here a complete tabulation such as has been given for strain 15 serum (table 5). It may be stated, however, that absorptions with each of these sera were conducted and controlled in the same way as for strain 15 serum. It would seem sufficient for the purpose of tracing specific inter-relationships to give only the approximate homologous agglutinin absorption percentages of each of the fifty strains for each of the sera. These results are presented in such a way in table 6 that the specific affinities of the strains may be readily evaluated. It may be stated again that our definite end point, as described on page 312, served as the agglutination titer indicator, that the homologous titer of the serum strain was always determined in connection with each absorption, and that a control absorption with meningococcus was also run with each serum.

In an analysis of table 6 let us first compare the results with the sera 104, 217 and 218, immune respectively to strains 15, 29 and 33. These strains were considered very closely related as the homologous agglutinins produced by each were absorbed by each. The table shows that the specific or homologous agglutinins of these three sera are absorbed in a percentage amounting to 75 or over by twenty-two of the fifty strains. These twenty-two strains (3, 4, 6, 8, 9, 10, 11, 15, 19, 23, 29, 30, 31, 32, 33, 34, 35, 36, 37,

42, 43, 50) may be placed together in one group as far as the results with these sera are concerned. The agglutinin content of these three sera is also similar in that five strains (2, 16, 27, 41 and 49) are incapable of absorbing any of their respective specific agglutinins and closely associated with these are five other strains (5, 25, 28, 39 and 46) which show a specific relationship, and that only partially, to only one of these sera, namely, strain 15 serum. It should be mentioned that the gonococcus strains had been under cultivation for a much longer period at the time of strain 15 serum absorptions than when the tests with the other two sera were made. This, as will be explained later, is an important consideration due to the labile nature of the antigenic constituents of some strains. We have also a residuum of eighteen strains (1, 7, 12, 13, 14, 17, 18, 20, 21, 22, 24, 26, 38, 40, 47, 48) which show partial relationships to the above three serum strains in that they absorbed the specific agglutinins completely or incompletely from one or more of these sera.

Strain 8 showed marked specific relationships to strains 15, 29 and 33, and it would seem reasonable to expect that absorptions of a serum produced with this strain would indicate a similar grouping of the gonococcus strains. This, in large measure, proved to be the case, although there were rather fewer strains in the group absorbing little or none of the homologous agglutinins. With respect to a few strains, also, there are certain marked differences, as for example the fact that strains 27, 28 and 39, which absorbed little or none of the homologous agglutinins from strains 15, 29 and 33 sera, removed practically all of these agglutinins from strain 8 serum; on the other hand, strains 7, 12, and 44 did not absorb the homologous agglutinins from strain 8 serum but did so from the other three sera.

The next serum (106) reported in this table was immune to a strain (42) which showed very close relationships to the four preceding serum strains (29, 33, 15, 8). This strain produced a serum from which the homologous agglutinins were absorbed by a larger number of the test strains than was the case with any other serum investigated. There are thirty-three strains in the 75 to 100 per cent class and only eight in the 0 to 33 per cent

TABLE 6

Percentage absorptions of homologous agglutinins from nine gonococcic sera by 50 gonococcus strains and a meningococcus strain. Geographical source of serum strains: 29, U. S. A.; 33, U. S. A.; 15, foreign (?); 8, Mexico; 42, U. S. A.; 7, Germany; 18, England; 41, Belgium; 5, England

ABSORPTION PERCENTAGES	STRAIN 29 SERUM (217) HOMOLOGOUS TITER: 1000–2500 ABSORBED AT 1:50	STRAIN 33 SERUM (218) HOMOLOGOUS TITER: 1000–2500 ABSORBED AT 1:25	STRAIN 15 SERUM (104) HOMOLOGOUS TITER: 6000–8000 ABSORBED AT 1:250	STRAIN 8 SERUM (225) HOMOLOGOUS TITER: 500–1000 ABSORBED AT 1:25	STRAIN 42 SERUM (106) HOMOLOGOUS TITER: 1500–2000 ABSORBED AT 1:25 and 1:50	STRAIN 7 SERUM (230) HOMOLOGOUS TITER: 2000–3000 ABSORBED AT 1:50	STRAIN 18 SERA (122 and 237) HOMOLOGOUS TITER: 400–500 ABSORBED AT 1:25	STRAIN 41 SERUM (223) HOMOLOCOUS TITER: 500–1000 ABSORBED AT 1:50	STRAIN 5 SERUM (95) HOMOLOGOUS TITER: 500 ABSORBED AT 1:25
75 to 100	3, 4, 6, 8, 9	3, 4, 6, 7, 8, 9	1, 3, 6, 8	6, 8	1, 4, 6, 7	3, 7, 8	1	1, 8	2, 4, 5, 7, 8
	11, 17, 19	10, 11, 12, 13, 15, 19	10, 11, 12, 14, 15, 18, 19	10, 14, 15, 18, 19	10, 11, 15, 16, 18, 19	11, 15, 17	14, 17, 18	14, 17, 18	14, 16
	23, 26, 29*	20, 21, 22, 23, 24, 29	23, 24, 26, 29	20, 21, 24, 27, 28, 29	20, 22, 25, 26, 28, 29	25, 29	20, 28	24	21, 22, 24, 27
	30, 31, 32, 33, 34, 35, 36	30, 31, 32, 33, 34, 35, 36, 37	30, 31, 32, 33, 34, 35, 36, 37	30, 32, 33, 37, 39	30, 31, 32, 33, 34, 35, 36, 37, 38	33, 34, 37		30, 34, 36	30, 34, 35, 36, 37, 38, 39
	42	42, 43, 47	42, 43, 44	42, 43, 45, 47, 48	41, 42, 43, 45, 46, 47, 49	42, 48		41	40, 47, 48
	50	50	50		50				
50±	7, 10	1	4, 5, 7	1, 2, 3, 4, 5	8		4, 7, 8	6, 7	1, 6
	13, 15, 17	17		11, 13, 17	14	12, 14, 16, 18, 19	11, 12, 13, 15, 16	16, 19	11, 12, 13, 15, 19
	20		25, 28	23	21, 23, 24, 27	20, 24	23, 24, 26	21	23, 29

50±	37, 38	38	38, 39	34, 35, 36, 38		30, 35, 39	30, 32, 35	31	31, 33
	40, 43, 44, 45, 47, 48	40, 48	40, 45, 46	40, 41, 46, 49	44	45, 47	41, 49	44, 45	44, 46
				50					50
0 to 33	1, 2, 5 12, 14, 16, 18	2, 5 14, 16, 18	2 13, 16, 17	5, 7 12, 16	2, 5 12, 13, 17	1, 2, 4, 5, 6 10, 13	2, 5, 6 10, 19	2, 3, 4, 5, 9 10, 11, 12, 13, 15	3 10, 17, 18
	21, 22, 24, 25, 27, 28	25, 26, 27, 28	20, 21, 22, 27	22, 25, 26		21, 22, 23, 26, 27, 28	21, 22, 25, 27, 29	20, 22, 23, 25, 26, 27, 28, 29	20, 25, 26, 28
	39	39		31	39	31, 32, 36, 38	31, 33, 34, 36, 37, 38, 39	32, 33, 35, 37, 38, 39	32
	41, 46, 49	41, 44, 45, 46, 49	41, 47, 49	44	40	40, 41, 43, 44, 46, 49	40, 42, 43, 44, 45, 46, 47	40, 42, 43, 46, 47, 48, 49	41, 42, 43, 45, 49
	M†	M	M	M	M	50 M	50 M	M	M

* Absorption with homologous strain not always carried out, but where tested always 100 per cent.

† M = Meningococcus.

group. In the light of these findings we have concluded that this strain 42 is as nearly representative of the entire gonococcus group as any which we have encountered. This conclusion is substantiated to a considerable extent by the complement fixation tests reported in a following section.

Strains 7 and 18 show only partial relationships to those used in producing the five preceding sera; strain 7 absorbed strongly the homologous agglutinins from sera 218 and 106, partially those of 217 and 104, and none from 225; strain 18 absorbed completely or nearly so from 104, 106 and 225 sera, and not at all from 217 and 218. Sera produced with each of these two strains (7 and 18) indicated also that their agglutinogenic constituents differ markedly from those of strains 8, 15, 29, 33 and 42 in that their homologous agglutinins are absorbed strongly by relatively few of this collection of gonococcus strains. Yet, on the other hand, these two strains interabsorbed in too slight degree to warrant placing them in the same group. Strain 41 is also one which showed a close relationship to only a few of the other forty-nine gonococcus cultures. Of the seven preceding sera in table 5 this strain 41 absorbed completely only homologous agglutinins from strain 42 serum, and only nine heterologous strains caused a 75 to 100 per cent absorption of the homologous agglutinins from its own serum (223). Some of these strains absorbing strongly from strain 41 serum bring this culture into close relationship with strain 18. Strains 1, 14, 17, 18, 24 and 41, in fact, may be said to constitute a small group, but one which is not sharply separated from the main group of which strains 8 and 15 are examples.

The absorption tests with strain 5 serum (95) are of much interest in that this strain seems to serve as a connecting link between such aberrant strains as 2, 16, 21, 22 and 27, which failed largely to absorb from the preceding sera of table 5, and certain members of the main group such as 8, 30 and 34. Strain 5, as is shown in this table, is one which failed almost completely to absorb the homologous agglutinins from the eight other sera, yet the homologous agglutinins of its own serum were bound strongly by twenty of these strains. This is an example of a

phenomenon frequently observed in this study, namely, that the agglutinin-binding and the agglutinogenic properties of a given strain may exhibit apparent inconsistencies. Meinicke, Joffe and Flemming (15) noted a somewhat similar state of affairs in their study of serological relationships of cholera vibrios and ascribed the failure of certain vibrios to bind the homologous agglutinins produced by other strains to loss of avidity of those particular receptors as indicated by *in vitro* absorption tests, but *in vivo* these receptors regain their avidity and in conjunction with the other elements constituting the specific antigen of the vibrio may produce agglutinins for the entire group. It is conceivable, in like manner, in relation to certain of these gonococcus strains, of which strain 5 is an example, that the antigenic constituents may have become so altered as to impair their agglutinin-binding capacities and yet leave their agglutinogenic properties intact.

The absorption results with the nine sera reported in table 6 and also of two others, indicated to us that our strains could not be distributed among a number of distinct serological types which were sharply separated from one another, as is the case, for instance, as regards the first three types of pneumococci. There would seem to be one large basal group, the members of which are closely related but not all entirely identical from an antigenic standpoint. To this main or regular group, thirty-nine of our seventy-seven strains may be referred, namely, 3, 4, 6, 8, 9, 10, 11, 15, 19, 23, 26, 29 to 37, 40, 42, 43, 45, 47, 50 to 53, 60, 61, 63, 64, 66, 69, 70, 71, 72. Some of these strains embody antigenic elements of a highly generalized character and are thus representative of a considerable part of the gonococcus group. Strains 8, 15, 34 and 42 are examples of cultures possessing markedly generalized antigens.

Bordering on this main group, we have encountered sixteen strains of which the antigenic elements are more specialized but which, nevertheless, showed a close relationship to certain members of the main group. Such strains (as examples may be cited 5, 7, 12, 13, 14, 21, 24, 38, 39, 44 of table 6) may be designated, "intermediate," in view of their position between the main group and the following variant strains.

Finally we have encountered in this collection a considerable number of strains (19) which are more definitely separated from the main group and which exhibit individual antigenic variations to a marked degree. We would designate these as "irregular strains" and place the following in this class: 1, 2, 16, 17, 18, 20, 22, 27, 28, 41, 46, 49, 55, 62, 67, 72, 75, 76, 77. Some of these strains, as 1, 17, 18, 20, and 41, as already stated, showed close enough relationships to warrant, perhaps, placing them together in a small group, although strain 1 tended later to develop the qualities of a regular strain. It is quite possible that through the use of other sera further relationships among certain of the irregular strains might have been discovered. In fact, as shown in table 6, a relationship to some one of the regular strains has been demonstrated for a number of these irregular strains, indicating that they may possess certain antigenic elements in common with the main group. We may, perhaps, conceive these variants as having undergone a rearrangement of their generalized antigens through adaptation to differing environments to such a degree that certain strains, such as 2 and 22, seem to be almost distinct serological entities. As will be indicated presently, however, it is conceivable that they all may be capable of reversion to a type approximating the original pattern.

In classifying the gonococci under the three general headings of (*a*) regular, (*b*) intermediate and (*c*) irregular strains, we mean to convey our impression that the antigenic constitution of these organisms is so variable and so prone to individualistic expression that the supposition of the existence of sharply defined types or groups is not warranted. This conclusion seems to us to be especially justified by the fact that the gonococcus antigen has exhibited marked tendencies to lability. This point will be discussed later in detail.

The most extensive serological study of the gonococcus group, based on the absorption of homologous agglutinins, which has been published within recent years, is that of Hermanies (13). Jötten (16) differentiated four different groups into which twenty of his twenty-seven strains apparently fell, but he does not report having used absorption methods in his classification, so our

results may not be compared with any degree of assurance with his. Hermanies' work constitutes a painstaking and close analysis of eighty-five gonococcus strains and his results are of much interest. Through the use of agglutinin absorption methods, he concluded that his eighty-five strains might be distributed among six immunological types, which he believed to be distinct and clear-cut. His group 2, consisting of thirty-six strains, was further divided into four sub-types. In his first group were included the ten so-called "Torrey strains." In a personal communication he has informed us that one set of these strains was obtained from the New York (City) Department of Health Laboratories and as our set of these strains was obtained from the same source, we may be reasonably sure that his group 1 of forty-one strains and our main group of thirty-nine regular strains correspond as, in both instances, these groups include these "Torrey strains." His second group of thirty-six cultures included strains which varied greatly in their agglutinogenic and absorptive capacities. We have not been able to detect any second large group among our series of strains, although some of our intermediate or possibly the small group of five irregular strains might be found to correspond to certain of his group 2 types. His other four types were made up of more or less isolated variants; three strains constituting his type 3, two his type 4, and one each in his types 5 and 6. It is quite possible that some of our irregular strains may correspond to these latter types.

In comparing our results with those of Dr. Hermanies and in attempting to reconcile the differences, certain considerations should be held in view, all or any of which may be accountable in some measure for the dissimilarity of our conclusions. In the first place our stock cultures have been maintained almost from the time of isolation on a semi-solid serum-free medium, whereas the Hermanies strains were carried on ascitic agar slants. It is possible that our medium is more likely to bring to light generic interrelationships than is the solid ascitic agar. Again, many of our strains were derived from widely separated geographical localities, the infection having been contracted in

a considerable number of instances in foreign countries, and as regards other cases in widely separated parts of this country. Of the foreign strains, three are from England (2, 5, 18), six from France (4, 6, 46, 66, 69, 73), two from Mexico (8, 74) and one each from Germany, Belgium, Egypt and Panama (respectively, 7, 41, 53, and 76). A number of other strains obtained from Dr. E. Finch are probably of foreign origin, but their history is not definitely known. The majority of the Hermanies strains are described as having been isolated from patients in the Cincinnati General Hospital. It is, perhaps, reasonable to suppose that such a comparatively restricted environment would yield a larger number of strains of a similar type. And, finally, our methods for conducting the absorption tests were quite different. It seems probable that we absorbed with a larger dosage of gonococci than did Hermanies and perhaps with a more uniform dosage. His technic was based on the assumption that ascitic agar slants of fairly constant area would yield a like amount of growth with each of his gonococcos strains. It has been our experience that various gonococcus strains may differ markedly in the amount of growth produced on a given lot of medium and that comparative absorption tests resting on the supposition that growth obtained from approximately equal areas of culture medium surface will prove at times misleading. Further, in the Hermanines technic absorption tests were confined to the determination of the degree of removal of the homologuos or specific agglutinins but no check was made on absorptions of the group agglutinins. It is obvious that if these are not absorbed completely they might influence the titer obtained in tests of the absorbed serum with the homologous strain. There is no statement, also, in regard to the selection of a definite end point for the agglutination reaction. In comparative work this would seem to be a matter of prime importance.

A careful study of the Hermanies tables would seem to indicate here and there some relationships between his several groups. Certain of his type 1 strains absorbed at least 50 per cent of the specific agglutinins from a type 5 serum and also a type 4 strain apparently absorbed 75 per cent from type 5 serum. In his

second article (14) table 6, there is an indication that three type 1 strains and also the type 4, 5 and 6 strains removed at least 50 per cent of specific agglutinins from a type 2(b) serum; in the same article, table 8, six other type 1 strains absorbed 50 per cent or over of type 2(a) agglutinin from a type 2(b) serum. A few other instances of apparent cross absorptions might be cited, all of which indicate that there existed at least some degree of inter-relationship between these Hermanies types. It seems to us quite likely that if antisera had been produced to a considerable number of the type 1 strains, certain ones might also have been found occupying an intermediate position between the type 1 and type 2 groups.

In his second article (14) Hermanies has analyzed his thirty-five type 2 strains and demonstrates that they may be divided into four fairly distinct and characteristic sub-types—the *a*, *b*, *c* and *d* races. The matter was futher complicated by the finding that the individual strains of these several races differ more or less among themselves in the relative distribution of these several agglutinogens. There exists, then, in this group a very complex state of relationships. The point, however, which we wish to emphasize particularly at this place is that these several agglutinogenic components, according to Hermanies' findings, may change in relative amount under cultivation; for example, as regards his strain 66, he found that after a period of cultivation the antigenic component designated as b had become much reduced with a coincident augmentation of the so-called *x* moiety. Other similar instances of marked lability of the antigenic constituents of this group are noted in his article.

Very recently Cook and Stafford (31) have reported, as a result of an agglutinin absorption and alexin fixation study of sixteen gonococcus strains, that they were not able to define any groups, absorption of agglutinins having taken place without uniformity. The technic employed in the absorptions was essentially that given by Hermanies.

In 1907 one of us (1) determined that six of the ten gonococcus strains studied might be distributed through agglutinin absorption experiments among three groups which were apparently

serologically independent of one another. In reëxamination of five of these strains (one was not available) we were much surprised to find that they had become very closely related as

TABLE 7

Experiment to show the changes in absorptive capacity which have occurred in certain "Torrey strains" after fourteen years cultivation. Set 1 = strains obtained from the New York City Health Department Laboratories. Set 2 = strains obtained from H. K. Mulford Company Laboratories. Set 3 = strains obtained from Parke, Davis and Company Laboratories. Test serum for 1921 absorptions immune to strain 36 (original G) of set 1. This serum was absorbed with each of the strains of each set and then tested with the homologous strain. Titer, 3000. Absorbed at 1:100

"TORREY STRAINS" (ABSORBING STRAINS)	ABSORPTIONS OF 1907		ABSORPTIONS OF 1921 WITH					
			Set 1 strains		Set 2 strains		Set 3 strains	
	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum	Titer with unabsorbed serum	Titer homologous strain with absorbed serum
A	800	2000 (0)*	2000	<200 (100)	1000	1000 (66)	1000	500 (84)
B	400	Not tested	2000	200 (94)	500	200 (94)	2000	200 (94)
C	800	2000 (0)	3000	<200 (100)	2000	200 (94)	2000	1000 (66)
G	2000†	–	3000	<200 (100)	4000	500 (84)	200	500 (84)
H	2000	800 (60)	–	–	–	–	1000	1000 (66)

* Figures in parenthesis indicate percentage absorptions of homologous agglutinins.

† Homologous.

regards their antigenic constituents. In table 7 there is presented a comparison of the absorption results with strain G (36) serum in 1907 and 1921. It may be observed that in 1907, strains A (strain B was closely related to A) and C absorbed none of the specific agglutinins from strain G serum, but strain H

absorbed 60 per cent. In 1921, however, we find that three sets of these strains obtained from three independent laboratories (H was available from only one source) had approached a common type in that all the A, B and C strains now absorbed from two-thirds to all of the homologous agglutinins from a strain G serum. It is of interest to note that, whereas a variety of media had been used in the cultivation of sets 1 and 2 during this fourteen-year period, set 3 had been maintained on an ascitic agar medium of uniform character.[4]

Other similar experiments in which a strain B (30) serum was absorbed with these three sets of cultures have given similar results, in that strains C and G, which in 1907 appeared quite distinct from strain B serologically, now absorb very strongly the specific agglutinins for strain B. Hermanies has placed all of the "Torrey strains" received from two sources in his type 1 group, although six of the ten strains received from another source constitute his *c* race of type 2. We also have found nine of these strains (A, 29; B, 30; C, 31; L, 32; 0, 33; S, 34; Q, 35; G, 36) sufficiently alike serologically to warrant placing them together in our group of regular strains. It is evident, then, that gonococcus strains which at the time of isolation are apparently distinct serological entities or belong to particular groups, may tend after a period of cultivation to assume a common generalized antigenic condition. We are also convinced that antigenic lability should not be postulated as confined to one particular group or type, as assumed by Hermanies, but is rather a generic characteristic shared in varying degree by all gonococcic strains.

Absorptions with twelve selected strains

Our inability to demonstrate the existence of distinct and stable types in the gonococcus family led us to approach the problem of serological relationships among these organisms from a different standpoint, namely, the application of absorption tests to a large number of different strain sera with a selected set of twelve

[4] Set 2 was composed of strains which had been received from the New York Health Department Laboratory two to eight years previously.

strains in order to single out one or more strains of such a generalized character as to be fairly representative of the whole gonococcus group. An advantage pertaining to making absorption tests with such a limited number of strains lies in the fact that an experiment with a given serum may be carried out in a single day and thus under more uniform conditions than when the tests are extended over several days as is necessary with a large collection of strains.

These twelve gonococcus strains were in some measure representative of the principal serological variants encountered among the first fifty strains examined. The twenty-seven monovalent sera which were absorbed, were immune to strains representing a still wider range of variation. In table 8 are collated the results obtained in this comparative series of absorptions. The figures under each serum represent the percentage of absorption of the homologous agglutinins which was effected by each of the several selected strains. In the case of a few sera an absorption was not carried out with the homologous strain but it may be assumed that 90 to 100 per cent of the agglutinins would have been removed. With reference to anti-sera to strain 5 and also to 29, the results with two sera produced at different times are given in order to illustrate fluctuations in absorptive capacities which were observed in certain strains—such as 1, 7, 18, and 25—under conditions of artificial cultivation. These experiments were all conducted and controlled in the manner illustrated in table 5, but for our present purposes it is sufficient to report the data in regard to the percentage of absorption of the homologous agglutinins.

Table 8 shows clearly that there exists a diversity in the range of the absorptive capacities of these selected strains. In order, however, that these differences may be readily evaluated, a graph is presented giving the percentage absorption value of each strain. In computing these percentage values, a score of 2 points was given for a practically complete absorption of the homologous agglutinins (75 to 100 per cent); for a partial absorption (about 50 per cent) 1 point, and a slight absorption (not exceeding 33 per cent) was counted as negative. On this

TABLE 8

Absorptions of 27 gonococcic sera with 12 selected gonococcus strains and 1 meningococcus strain. The figures indicate the percentage absorption of homologous agglutinins

	GONOCOCCIC SERA; SERUM STRAIN NUMBER FIRST AND RABBIT NUMBER IN PARENTHESES																												
	1 (219)	2 (247)	5 (251)	5 (121)	8 (225)	11 (118)	13 (861)	14 (103)	15 (104)	18 (122)	19 (119)	25 (125)	27 (81)	28 (82)	29 (217)	29 (236)	30 (236)	33 (218)	34 (116)	36 (127)	38 (105)	41 (235)	42 (106)	46 (83)	48 (227)	49 (129)	53 (107)	56 (108)	64 (239)
1	H	0	50	25	50	33	50	100	87	80	100	88	33	50	0	94	97	0	33	100	100	80	75	50	80	87	25	84	80
7	50	50	90	50	0	95	50	66	50	50	100	88	75	0	50	88	50	75	93	33	94	90	100	0	50	33	50	84	0
						H																							
11	80	0	50	25	50	95	80	66	100	50	90	100	75	0	50	88	84	95	93	66	66	50	95	50	100	66	88	84	94
									H																				
15	0	50	50	50	90	95	50	66	100	50	100	100	0	0	50	88	33	100	93	100	84	50	95	50	100	66	88	66	88
										H																			
18	50	50	0	50	100	0	0	100	75	100	0	100	0	0	0	0	0	0	0	66	84	100	95		80	66	50	84	80
											H																		
19	0	0	50	75	90	95	80	94	93	0	90	100	0	0	75	88	100	100	93	83	100	80	75	0	50	87	50	66	100
												H																	
25	80	50	0	0	90	33	50	33	100	0	75	100	33	50	0	88	0	90	93	66	100	20	100	0	100	94	88	84	0
																	H												
30	0	0	80	100	100	95	80	94	100	0	90	88	0	0	100	100	100	100	100	93	100	40	75	0	50	87	0	84	88
34	0	50	80	75	90	95	80	94	75	0	100	100	75	0	90	100	100	100	H	83	100	80	100	100	100	66	75	84	88
38	80	50	80	90	50	95	80	66	50	0	75	50	87	0	50	63	33	50		0	H	0	95	50	0	66	50	66	94
																						H							
41	100	0	0	0	50	0	80	100	0	50	0	50	33	50	50	63	0	0	66	33	100	100	95	0	0	66	100	33	25
																										H			
49	80	0	0	0	0	50	100	33	0	50	0	75	0	50	0	0	0	0	83	0	100	0	95	50	100	100	75	50	50
Meningococcus	0	0	0	0	0	33	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0

Homologous titers and absorption dilutions of above sera

Homologous titer	500	250	500	2000	500	2000	500–1000	3000	8000	500	1000	200	750	500	1000	8000	3000	1000	3000	3000	3000	500	2000	250	250	2000	2000	600	800
Absorption dilution	1:25	1:50	1:25	1:100	1:25	1:50	1:50	1:100	1:250	1:25	1:50	1:12.5	1:25	1:25	1:50	1:250	1:50	1:25	1:100	1:100	1:100	1:25	1:50	1:25	1:25	1:50	1:50	1:12.5	1:25

basis, then, the highest possible score for the twenty-seven sera would be 54.

This graph shows clearly the fact that of these particular strains, 34 possesses the widest and most generalized absorptive capacities, effecting a marked absorption of the homologous agglutinins from twenty-four of the twenty-seven sera. This

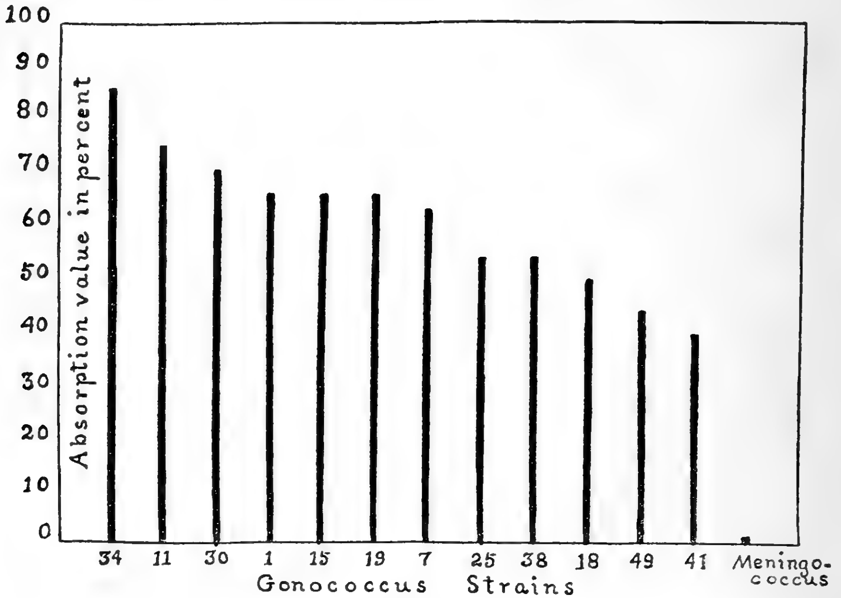

CHART 1

strain, as has already been mentioned, is one of the set obtained from the New York Health Department Laboratories under the designation "S." Strains 11 and 30 proved to be next in efficiency in absorptive capacities with the others grading off to those with the least generalized absorptive properties, namely, strains 41 and 49. We do not wish to convey the impression that strain 34 is unique among our collection of cultures in its generalized affinities for it seems highly probable that certain other strains, such as 8 and 42, might have proved quite as effective.

General observations in reference to table 8

Tolluch (17) in discussing the importance of the application of standardized agglutination methods in a study of immunological types of a given bacterial species, mentioned the desirability of using sera with as uniform a titer as possible. Other investigators, such as Gordon (18) for meningococci and Hooker (7) for the typhoid bacillus, have reported the presence of greater specificity in sera from rabbits after a few inoculations than after many; or as Gordon has expressed it, in the "first born agglutinin." Although these general principles may apply to the gonococci, it was not found feasible either to restrict the number of inoculations to a small number or to attempt to employ sera with a nearly uniform titer. This was due to the fact that the rabbits responded very unequally to inoculations with various strains. With some strains a fairly high titer could be obtained after six inoculations given in a period of two weeks, but with other strains a prolonged course of immunization was necessary before a serum could be obtained with a titer perhaps only one-half or less as high as with the former.

As has already been mentioned, relatively inagglutinable strains were encountered more frequently than was anticipated. Inagglutinability, however, does not necessarily mean the absence of agglutinogenic or agglutinin-absorbing capacities as was demonstrated by McIntosh and McQueen (19) some years ago in a study of an inagglutinable typhoid strain, and more recently in reference to typing meningococci by Mathers and Herrold (20) and others. On the other hand Bemains (21) has shown that a strain of *B. dysenteriae*, which was artificially rendered inagglutinable, also lost its agglutinogenic and agglutinin absorbing functions. It might be expected that the relatively inagglutinable strains of gonococcus would also prove poor absorbers of agglutinin, but this, although true in some instances, was not always the case; certain poorly agglutinating strains, such as 25 (table 8) absorbed agglutinins strongly and consistently. Another similar point, which has already been discussed, is further illustrated in table 8, namely, that the agglutinogenic and the absorptive capac-

ities of a strain may not run entirely parallel. For example, strain 38 produced a serum from which the homologous agglutinins were absorbed in greater or less degree by all these twelve selected strains and yet strain 38 removed none of the homologous agglutinins from sera produced respectively with strains 1, 18 and 30. Other instances are provided by strains 27 and 49, both of which absorbed agglutinin poorly and were apparently unrelated to other larger groups of gonococci but which, nevertheless, produced agglutinins readily absorbable by regular types. In determining serological relationships among the gonococci, then, it is not sufficient alone to absorb other sera with a given strain but a serum should be produced with this strain and absorbed in turn with other typical strains as it is evident that in some instances the function of binding agglutinin may have become inactivated or suppressed in some degree without affecting the agglutinogenic propensities.

Teague and McWilliams (22), in a study of the effect of spontaneous agglutination on the absorption of agglutinins, found that in the case of a typhoid strain spontaneously agglutinating subcultures absorbed less agglutinin than did the normal sub-cultures. They inferred from this finding that physical aggregation may play a part in effecting the amount of agglutinin absorbed by various strains. Gonococcus strains with tendencies to spontaneous clumping have been encountered not infrequently in this work but this tendency has not seemed to interfere with agglutinin absorption, perhaps because the aggregations were more readily broken up than is the case with flagellated bacilli such as *B. typhosus*.

Vulvovaginitis strains

This series of gonococcus cultures included thirteen strains isolated from cases of vulvovaginitis in young girls. The ages of these patients ranged from two to eleven years, with ten under six years. These strains were studied with particular care in order to determine if they constitute a type immunologically distinct from those recovered from adult gonorrheal infections. Louise Pearce (23), from a comparative serological study of six

infant strains and nine strains from adult cases, came to the conclusion that there are inherent differences in the types of gonococci causing these two classes of infections, although not to an extent which would warrant separating them into two distinct immunological groups. Her methods of investigation included agglutination tests without absorptions and complement fixation experiments. The results with these two methods were harmonious. It is obvious that if the gonococcal strains recovered from these two classes of infection could be shown to be representative of serologically distinct types, it would clarify our ideas in regard to their epidemiological relationship.

In table 9 the results of absorptions with sera immune to two of these vulvovaginitis strains are reported and in table 8 the absorptions of these sera (108 and 239) with twelve adult strains. In table 5 are detailed further the absorptions of a serum (104) immune to an adult strain (15) with these infant case strains.

These several cross absorptions indicate clearly that no definite serological distinction may be drawn between infant and adult strains. Among these infant strains, as is demonstrated in table 9, one encounters the same lack of homogeniety as to type as would be observed among a similar series of strains isolated from adult cases. We also find among the infant strains certain ones (48, 60, 61, 63, 64) which absorbed strongly the homologous agglutinins from an adult strain serum (table 5) and also others (55, 56, 57, 62, 75) which removed little or none of such agglutinins from this adult, regular strain serum. One fairly definite characteristic, however, was noted in the study of these infant strains and that was that their agglutinogenic potentialities were not strong. No serum with a titer above 1:800 was obtained with any of the four strains employed in rabbit immunizations. With one strain (48) the titer could not be forced above 1:250 in spite of a prolonged series of inoculations of large dosage. It should be noted, however, that the production of low titer sera was not confined to infant strains as certain adult strains, especially of the irregular type, exhibited the same tendency, but it appeared to be more of a distinguishing trait of the infant strains. These infant strains also, as a class, tended

to absorb agglutinins rather less strongly than most of the adult strains. This weakness in antigenic properties may be a factor in determining the mild yet chronic course which most of

TABLE 9

Two sera immune to vulvovaginitis strains (children cases) 64 and 56, absorbed with other strains from the same type of cases.

STRAINS	TITER WITH UNABSORBED SERUM	TITER ABSORBING STRAINS WITH ABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH UNABSORBED SERUM	TITER HOMOLOGOUS STRAIN WITH ABSORBED SERUM	PERCENTAGE ABSORPTION OF SPECIFIC AGGLUTININS
Strain 64 serum, titer 800; absorbed 1:25					
55	50	<50	800	100	88
56	<50	<50	800	800	0
57	<50	<50	800	400	50
58	200	<50	800	200	75
59	400	50	800	100	88
60	400	100	800	200	75
61	200	50	800	100	88
62	100	<50	800	400	50
63	800	100	800	800	0
64*	800	100	800	100	88
65	100	<50	800	800	0
75	400	<50	800	400	50
Meningococcus	<50	<50	800	800	0
Strain 56 serum, titer 500; absorbed at 1:25					
48	100	<50	500	50	90
54	750	100	500	250	50
56*	500		500		
58	500	100	500	100	80
60	1000	<50	500	250	50
61	1000	<50	500	250	50
62	100	<50	500	250	50
64	750	100	500	100	80
Meningococcus	<50	<50	500	500	0

* Homologous.

these cases run in that insufficient stimulus is provided for the production of an adequate amount of bactericidal antibody on the part of the host.

It should be observed that the majority of these vulvovaginitis strains were isolated from patients under treatment at a clinic

(Vanderbilt Clinic, through the courtesy of Dr. B. Wallace Hamilton) and it was thus entirely unlikely that the infection could have been received in any instances from a common source. On the other hand, if such strains are isolated from cases occurring in an institution, there is always a possibility that a single strain may have been spread through contact and, accordingly, a study of such cultures might give an unwarranted impression of serological homogeniety as existing among gonococci causing these vulvovaginitis infections.

DISCUSSION

Within recent years much time and effort has been devoted by various investigators to the detection and formulation of distinct immunological types among different species of pathogenic bacteria, the members of which are culturally identical or nearly so. Without doubt these efforts, in some instances, have served a very useful purpose. There is, however, the lurking hazard in extending this method of grouping to other bacterial species that one may depart widely from a natural system of classification. A type or group, if established on a rational basis, should represent a distinct serological entity. The antibodies of a serum produced with a member of a given type should be absorbed completely, or nearly so, by all strains referred to the same type but should not be affected by strains assigned to a different type. Or, as Eastwood (24) has expressed it in an illuminating discussion of this question, "if strains belonging to the species fall into distinct groups without cross-division, when tested by a number of monovalent sera, grouping is indicated; but if such tests produce marked cross-division, grouping is not justifiable." To quote further, "where each main group is so elastic that its margin of separation from the others is small, one begins to raise the question whether the adopted system of grouping has turned out to be artificial and arbitrary, and whether the species under consideration is really amenable to subgrouping."

In these remarks, Eastwood was referring particularly to certain proposed groupings of the meningococci. Dopter's orig-

inal division of the meningococci into the true meningococci and the parameningococci was a natural one in that we have here two groups which are generally recognized as being serologically distinct, but in the further division of the meningococci into four groups in accordance with Gordon's classification (18), two groups have been added which, perhaps, are not founded on as sound a basis. In fact, a considerable number of investigators of the meningococci have reported that the Gordon group III is not sharply separated from I, nor IV from II; affinities which Gordon, himself, has recognized. Griffiths (25) and also Scott (26), from a study of spinal and nasopharyngeal strains derived from much the same sources as those of Gordon, both concluded that only two main groups should be recognized; a conclusion which Eastwood has considered justifiable. Griffiths has also inferred that "the two groups are not fixed types but may be further sub-divided by means of absorption experiments into sub-groups which are probably 'centers of variation' of the different stages of evolution of the meninogococcus antigens."

It is the opinion of the writers that there is much less justification for the formulation of distinct groups in the gonococcus than in the meningococcus family. Among the gonococci there are no two groups as sharply separated as the normal meningococci and the parameningococci. In fact, if agglutinin absorption and agglutinogenic properties of *each* strain are taken into consideration, as they really should be in an attempted formulation of types, we would find so many strains exhibiting individual immunological variations and also inter-relationships between what might, at first sight, be considered representatives of distinct types that the line of demarkation between the proposed groups would tend to become completely obliterated. We have, accordingly, on the basis of the evidence already presented, distributed our gonococcus strains under three general headings, namely, regular strains, the most generalized as regards antigenic properties, intermediate strains, which are quite closely related to certain of the regular types, and the irregular strains which exhibit marked individualistic variations. In employing these general terms we are adopting a mode of classification similar

to one which has been extensively applied to the meningococci, but in view of our findings, especially as regards the marked antigenic lability inherent in the gonococcus group, we feel that it is as definite a one as the conditions justify.

This phenomenon of antigenic lability, as exhibited by the gonococcus, is most interesting. As has been mentioned, Hermanies noted a marked degree of lability on the part of certain members of his type 2 group. During the course of our experiments we have observed numerous instances in which the antigenic constitution of strains have undergone some change under cultivation. By way of example reference may be made to the results obtained with two sera (217 and 236, table 8) immune to strain 29. An interval of over a year occurred between the times of production of these sera against a strain which had become stabilized. It may be noted, however, that strain 1 has, during that interval developed affinities very similar to the serum strain 29, a member of the regular group, although soon after isolation this strain 1 showed a close relationship to strain 41, a member of the irregular group (table 8). Strain 25, also, during this period has developed evidence of a close relationship to this serum strain, and strain 7 has passed from an intermediate position to a close affinity to the regular group. These are only a few instances, among many, in which there has occurred a change in antigenic responses to various sera. Beside such changes in these comparatively recent strains, we have the still more striking evidence of antigenic lability as presented by the changes which have occurred in the "Torrey strains" during the fourteen years of cultivation (table 7). In the face of such a tendency to mutation in antigenic constitution on the part of gonococci, one would have no assurance that strains selected as type representatives might not change entirely in character after a period of cultivation. We believe, in fact, that the whole tendency under conditions of artificial culture is for reversion to our regular group and that a strain having attained that disposition of its antigenic components remains in a comparatively stable condition.

Changes in type under cultivation have also been noted by

various observers as occurring in certain strains of the meningococci. Griffiths, in fact, in view of the variations in antigenic characteristics observed during sub-cultivation concluded that "meningococcus antigens are not precisely fixed or stable substances, but are liable to modification under the influence of environment." He believed these changes might take one or the other of two directions; toward either increased or diminished complexity of the structure of the receptor apparatus, which would mean an increase or diminution in the range of binding capacity. Scott has likewise reported temporary variations in agglutination between different sub-cultures of the same strain of meningococcus, and also variability in the absorptive power of one strain. Both of these phenomena, however, he considered due to the presence of two varieties of cocci of differing sensitivity to agglutinin and absorptive powers within a single strain with sometimes one and sometimes the other predominating and not to antigenic changes within the substance of the cocci. These peculiarities, however, in the absorption of agglutinin and also the large number of serological varieties which he encountered, caused him to doubt the practical value of this mode of classification in defining types among the meningococci. Walker (27) has concluded in connection with a discussion of the significance of meningococcic types that "fixity of these (Gordon's) is not proved." Butterfield and Neill (9), although believing Gordon's type strains to be stable and finding sera prepared with them very effective in the classification of their series of meningococci, reported that certain strains during a year's time apparently changed from one type to another. A very interesting observation in this connection is that of Eberson (28) on the effect of ultra-violet rays on the antigenic properties of the meningococci. He found that by exposure to these rays, regular and irregular meningococci could be so altered that they developed the property of producing mutual agglutinins, but parameningococci could not be so altered. He further concluded from similar tests that regular types of meningococci include within their protein molecules the elements of the parameningococci but that the latter possesses no agglutinogenic radicle common to the regular and thus constitutes a distinct type or species.

In attempts to explain the development of serological variants among certain groups of bacteria, a number of different hypotheses have been offered. The theory of Meincke and his colleagues of the loss of avidity on the part of certain receptors of the cholera vibrio has already been mentioned. Eastwood (24) has suggested that a more reasonable explanation of these changes in antigenic behavior lies in the conception that antigen is "a chemical substance which may exist in one or other of several different chemico-physical phases, demonstrable *in vitro.* And the same conception would apply to antibody." He further suggests that these various chemico-physical phases are closely related to changes in stereo-chemical structure of the molecules of the bacterial protoplasm. This theory, of course, is at present highly speculative, but it may find confirmation through later advances in physical chemistry. Hermanies, in a discussion of the origin of the sub-groups and races which he encountered in his group 2 of gonococci, proposes the theory that his four types were originally derived from a common type containing alone the agglutinogen *x*, but under different environmental conditions this *x* moiety has tended to retrogress and has been replaced in greater or less degree by the antigenic elements *a*, *b*, *c* and *d*, according to the particular race to which a given strain is related. This process, he suggests, may be carried so far that the original *x* element becomes lost and a new species characterized by one or other of the four acquired antigenic elements may arise. The new species would seem to be fixed in type because, as the antigenic element *x* has been entirely eliminated, there is no possibility of reversion to that original type. In view, however, of our experimental results it seems to us that this theory assumes a greater degree of acquired antigenic stability than is exhibited by the gonococcus group. It is our opinion that as a result of adaptations of gonococcal strains to different environments, including the defensive agencies of the host, there have arisen varied molecular configurations or physical-chemical phases—to use Eastwood's term—of the same specific basal substance, none of which, however, attain a state of complete stability and thus do not give rise to new fixed types. Further-

more, as none of the original elements have been lost or eliminated, there may occur in time following exposure to an environment of fairly uniform type, such as is presented by a culture medium, a molecular redistribution of the antigenic substance until a pattern resembling that characteristic of our regular and generalized group is attained.

Griffiths (25) has suggested that enhanced virulence and invasive powers of the meningococcus may be correlated with increased complexity of the receptor apparatus; that is with an increase in range of combining capacity. From this point of view our most complex gonococcic strains are those designated as regular, with the intermediate next in order and the irregular types exhibiting the least degree of complexity. Although the majority of our strains were isolated from more or less acute cases of urethritis and accordingly their invasive propensities could not be evaluated, there were eleven strains in the series recovered from cases showing complications. Among these there was one case of ophthalmia, three of chronic prostatitis, one epididymitis, four joint infections and two septicaemias. Ten of the eleven strains isolated from these cases showing complications were of the regular or intermediate type. Although the number of these cases is too few for a definite conclusion, the tentative suggestion may be made that the irregular variant strains are less likely to give rise to complications than are those resembling the regular types. Jötten (16) found that his more virulent strains fell into two of his four groups, but as to whether the members of these two groups are serologically related to our regular types, we, of course, can offer no opinion.

COMPLEMENT FIXATION

In these complement fixation experiments with the gonococcus our purpose has been not so much to confirm the conclusions derived from the agglutination work in regard to the serological relationships of our strains as to apply the facts disclosed to the selection of strains most useful as antigens for the diagnostic complement fixation test. Incidentally, however, we have noted a marked degree of correspondence between the strain affinities revealed through these two methods.

One of the great obstacles to the general use of this test in the past has been the difficulties associated with the preparation of the antigen, especially as the delicate nature and apparent fastidious cultural requirements of the gonococcus have made their maintenance a troublesome matter and also because it has seemed necessary to employ a considerable number of strains in preparing the antigens. Commercial distributers of biological products, also, have been unsuccessful in supplying this antigen in an entirely satisfactory way because of its tendency to develop anti-complementary properties. It would seem, then, that if one or two readily cultivatable, representative strains should prove to be as efficacious as antigens as the combinations of the ten or more strains, which have been used generally heretofore, the test would be much simplified and hence more generally available.

TECHNIC

Antigen. The following serum-free solid medium has been used for growing the cultures for antigens. This medium has a growth accessory principle and is prepared according to the Huntoon (4) method, although the formula has been modified. It has generally yielded a luxuriant growth with the gonococcus strains employed.

Salt-free, 1.5 per cent peptone, "vitamine" agar. Five hundred grams of fresh chopped beef heart, free from fat, one whole egg and 1 liter of distilled water are placed in a double boiler over a free flame and the temperature is maintained at 60°C. with constant stirring for five minutes. Fifteen grams of peptone (Difco) and 18 grams of flaked agar are now added and the temperature raised until the medium assumes a brownish color. It is then made slightly alkaline to litmus (with a 10 per cent sodium carbonate solution) and is transferred to a flask, or better to a coffee pot, and heated in the Arnold sterilizer at 100°C. for two hours. The medium may now be cleared through centrifuging or by filtering through glass wool; if the latter procedure is used, the meat residue should be deposited on the glass wool in a funnel and the fluid portion allowed to perculate through; several times, if necessary. No cloth, cotton or other absorbent material

should be allowed to come in contact with it. After clarification, 2 per cent glycerine is added, the reaction is adjusted to pH 7.2, and the medium reheated. It is then distributed in potato tubes, sterilized in the Arnold and slanted. The slants should be laid down not longer than the day before they are to be used.

The large slants of this medium should be seeded from twenty-four hour growths on the same medium. They are then incubated for from twenty-four to forty-eight hours. In preparing the antigen the method of M. A. Wilson (29) was followed. The growth was well washed with 50 per cent and then 95 per cent alcohol, being allowed to stand one-half hour with each in a water bath at 37°C., with frequent shaking. After centrifuging, the sediment is covered with ether and allowed to stand at room temperature for one-half hour with frequent stirring. It is then centrifuged, the ether decanted off and the sediment placed over night in a dark place. The dry powder is then emusified in 0.85 per cent saline to the standard density and the proper dosage determined. It should then be heated at 80°C. for one hour, although in our experiments this heating was omitted as the antigens were prepared freshly at frequent intervals. The antigen may be preserved with 0.1 per cent phenol. Antigen prepared in this way yielded results superior to those obtained with filtered autolysates or with an antigen prepared according to Thomson's (30) method.

Gonococcic sera. Most of the immune sera were the same as those used in the agglutination experiments and, for the most part, had been preserved for a longer period of time. They were inactivated shortly before each test was performed.

Complement. The sera from three or four guinea-pigs were pooled and preserved by freezing. There was usually little or no loss in potency for four or five days. Only active complement, fixable by gonococcus, was used.

Hemolytic system. The anti-sheep system was used. One hemolytic unit of complement and two hemolytic units of amboceptor were employed. The complement was titrated immemediately before the test and the amboceptor at frequent intervals. The complement unit was read at the end of thirty

minutes in the water-bath at 37°C. Washed sheep corpuscles in a 2.5 per cent suspension were used, and the amboceptor and cells added separately.

The test. The total volume of the mixed reagents was adjusted to 2.5 cc. One unit of antigen was used and this was determined by titration with a polyvalent gonococcic serum or one with generalized affinities. This unit was the smallest amount of antigen which caused complete fixation of complement with 0.1 cc. of a 1:100 dilution of the gonococcic test serum. The antigen was diluted so that this unit was contained in 0.1 cc. This unit, of course, was always less than one-half the anti-complementary dose and generally much less. This titration was usually made just before the fixation tests were started. Controls were always prepared for the hemolytic system, for anti-complementary action of the immune sera and also the antigen, and also for the stability of the sheep cells. The tests were incubated at 37°C. for the usual periods and readings made at the end of one hour-incubation of the completed test and also after the tubes had stood over-night in the ice-chest. The last reading is the one which is recorded. In accordance with the usual custom, the symbol 4 indicates complete fixation; 3, almost complete fixation; 2, the hemolysis of about one-half the cells; and 1, only a slight degree of fixation.

COMPARATIVE TESTS

In table 10 are recorded the results of comparative tests of the fixation with twenty-seven monovalent gonococcic sera, with a polyvalent sera which was also used in titrating the antigens, and with a meningococcic serum. The four antigens employed in these tests consisted of the following single strains or combinations of several strains: First, strain 34; second, strains 15, 18, 34 and 41; third the ten "Torrey strains;" fourth, strain 42. In order to give full comparative value to the results, the four antigens were always tested on a given serum simultaneously and thus under exactly similar conditions. The object, as stated, was to determine whether such generalized strains as 34 and 42 yielded as good results as combinations of several

TABLE 10

Complement fixation with 27 monovalent and 1 polyvalent gonococcic sera and 1 meningococcic serum, using the following four gonococcic antigens: (1) Strain 34; (2) Strains 15, 18, 34, 41; (3) Ten "Torrey strains"; (4) Strain 42

SERUM STRAINS	ANTIGENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
2 (246)	1	4	3	3	3	2	1	—	—	—
	2	3	3	2	2	1	1	—	—	—
	3	4	3	3	2	1	1	—	—	—
	4	3	3	3	3	2	2	—	—	—
5 (121)	1	4	4	4	4	4	3	3	2	1
	2	4	4	4	4	4	3	2	—	—
	3	4	4	4	4	3	3	3	2	—
	4	4	3	3	2	1	—	—	—	—
7 (231)	1	4	4	3	2	1	—	—	—	—
	2	4	4	4	4	3	2	2	1	—
	3	3	3	3	2	1	—	—	—	—
	4	4	4	4	4	3	3	2	1	—
8 (243)	1	4	4	4	4	3	3	3	1	1
	2	4	4	4	3	3	3	2	1	—
	3	4	4	3	3	3	—	—	—	—
	4	4	4	4	3	3	3	2	—	—
11 (118)	1	4	4	4	4	3	3	2	1	—
	2	3	3	3	3	2	1	1	1	—
	3	3	3	3	3	2	1	1	—	—
	4	4	4	4	4	3	2	2	—	—
14 (103)	1	4	4	3	3	3	2	1	—	—
	2	4	4	4	3	2	1	1	—	—
	3	4	4	3	3	3	2	2	1	1
	4	4	4	4	4	4	4	3	2	1
15 (104)	1	4	4	4	4	4	4	4	3	3
	2	4	4	4	4	4	4	3	3	1
	3	4	4	4	4	4	3	3	3	3
	4	4	4	4	4	4	3	3	2	2
16 (87)	1	3	3	3	3	2	1	—	—	—
	2	3	2	1	—	—	—	—	—	—
	3	3	3	2	1	—	—	—	—	—
	4	3	3	1	—	—	—	—	—	—

TABLE 10—*Continued*

SERUM STRAINS	ANTI-GENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
18 (237)	1	3	3	1	1	1	1	1	1	1
	2	4	4	3	3	—	—	—	—	—
	3	4	4	3	3	2	2	1	1	1
	4	4	4	3	2	—	—	—	—	—
19 (119)	1	4	3	3	3	2	2	1	1	—
	2	4	4	3	3	1	—	—	—	—
	3	4	4	3	3	2	—	—	—	—
	4	4	4	4	3	2	—	—	—	—
25 (125)	1	4	4	4	4	3	3	3	2	2
	2	4	4	4	4	3	3	2	2	1
	3	4	4	4	4	3	3	3	2	—
	4	4	4	4	4	3	3	3	—	—
27 (81)	1	4	4	2	—	—	—	—	—	—
	2	3	3	1	—	—	—	—	—	—
	3	3	3	—	—	—	—	—	—	—
	4	3	3	3	3	2	2	—	—	—
28 (82)	1	1	1	1	1	1	—	—	—	—
	2	3	2	2	—	—	—	—	—	—
	3	4	—	1	1	—	—	—	—	—
	4	4	3	3	2	2	—	—	—	—
29 (236)	1	4	4	4	3	2	1	—	—	—
	2	4	3	3	3	1	1	1	1	—
	3	4	3	3	3	3	2	1	1	—
	4	4	4	4	3	2	1	—	—	—
30 (117)	1	4	4	4	4	4	4	3	—	—
	2	4	3	3	3	3	2	2	1	—
	3	4	4	3	3	3	2	2	1	—
	4	4	4	4	4	3	—	—	—	—
34 (128)	1	4	4	4	4	4	4	4	4	4
	2	4	4	4	4	4	1	—	—	—
	3	4	4	4	4	3	2	1	—	—
	4	4	4	4	3	1	—	—	—	—
36 (127)	1	4	4	3	3	2	1	—	—	—
	2	4	4	3	2	2	2	1	—	—
	3	4	4	4	3	3	2	2	1	—
	4	4	4	4	4	3	—	—	—	—

TABLE 10—*Continued*

SERUM STRAINS	ANTIGENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
38 (105)	1	4	4	4	4	3	3	3	3	2
	2	4	4	3	3	3	2	—	—	—
	3	4	4	4	4	4	3	2	1	—
	4	4	3	3	3	3	3	1	—	—
41 (238)	1	4	3	3	3	2	1	—	—	—
	2	4	4	4	4	3	3	2	2	—
	3	4	4	3	3	2	2	1	—	—
	4	4	4	3	3	2	2	2	—	—
42 (244)	1	3	3	3	2	—	—	—	—	—
	2	4	3	3	2	—	—	—	—	—
	3	4	4	3	3	1	—	—	—	—
	4	3	3	3	3	3	2	2	—	—
49 (129)	1	4	4	3	3	2	—	—	—	—
	2	4	3	2	2	—	—	—	—	—
	3	4	3	3	3	2	—	—	—	—
	4	4	4	4	3	—	—	—	—	—
53 (107)	1	1	4	4	4	4	4	3	2	1
	2	2	4	4	4	3	3	3	2	2
	3	2	4	4	3	3	3	3	3	3
	4	—	4	4	4	4	4	3	3	1
56 (108)	1	4	4	4	3	3	3	3	3	1
	2	3	3	3	3	2	2	1	1	1
	3	4	4	4	3	—	—	—	—	—
	4	4	4	3	2	1	—	—	—	—
62 (234)	1	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—
	4	3	3	2	2	2	2	—	—	—
64 (239)	1	4	4	4	4	4	4	3	3	3
	2	3	3	3	3	2	2	1	1	1
	3	3	2	1	—	—	—	—	—	—
	4	4	4	4	4	3	3	2	2	—

TABLE 10—*Concluded*

SERUM STRAINS	ANTIGENS	IMMUNE SERA DILUTIONS								
		0.05	0.01	0.004	0.002	0.001	0.00066	0.0005	0.00025	0.000125
74 (241)	1	4	4	2	1	1	1	1	1	—
	2	1	2	—	—	—	—	—	—	—
	3	3	3	3	1	—	—	—	—	—
	4	4	3	3	3	—	—	—	—	—
77 (245)	1	4	3	—	—	—	—	—	—	—
	2	4	3	—	—	—	—	—	—	—
	3	4	2	1	—	—	—	—	—	—
	4	4	3	1	—	—	—	—	—	—
Polyvalent 221	1	4	4	4	4	4	4	3	3	2
	2	4	4	4	4	4	3	3	3	2
	3	4	4	4	4	4	4	3	3	3
	4	4	4	4	4	4	4	3	3	3
Meningococcus (123)	1	3	2	1	—	—	—	—	—	—
	2	2	—	—	—	—	—	—	—	—
	3	3	—	—	—	—	—	—	—	—
	4	3	2	—	—	—	—	—	—	—

strains. The second combination was selected as these four strains seemed at the time to be fairly representative in their affinities of our series of gonococcus cultures. As the results show, however, they failed to cover the variations exhibited by our collection of strains.

The results in table 10 substantiate fully the conclusion which we reached as the basis of our agglutination experiments that our strains 34 and 42 are both markedly generalized in their affinities. Further it appears that an antigen prepared with either one of them yields on the average *better* results than those obtained with the combination of the ten "Torrey strains" (antigen 3) and also than with the four strains combined in antigen 2, although the latter was expected to exhibit a wider range of affinities. In the case of no serum was fixation obtained with multiple strain antigen 3 in which the single strain antigens 1 or 4 failed; on the other hand with nine sera stronger fixation occurred with antigen 1 than with antigen 3, and with eleven sera stronger with 4 than with 3.

In view of the above results, if a single antigen is employed for diagnostic tests, we would advise using either strain 34 or 42 alone, or perhaps in combination. It seems very questionable if better results would be obtained by combining a large number of regular and irregular strains in an attempt to produce a polyvalent antigen. In the first place it is questionable if such an antigen covering all the probable variants could be prepared; and in the second place when a large number of strains each with more or less limited affinities and representative of an equal number of so-called types are combined in a single antigen, then the antigenic elements for each of these types becomes so diluted that, in the dosage permissible, the effectiveness for each type would be greatly curtailed. Perhaps the best procedure would be to use two separate antigens in each test; one prepared with one or two representative strains with generalized affinities and good combining qualities, and the other with selected irregular strains.

Selected strains for diagnostic and therapeutic applications

For the preparation of a stock vaccine for use in cases for which an autogenous vaccine is not available, we would recommend a combination of our strains 15, 34 and 41. It seems probable, also, that these strains would serve as well as any in connection with the production of a curative serum.

Strains 15 and 41 in combination may be used to advantage in the production of an agglutinating serum with wide affinities which might prove helpful in the identification of gonococci.

For the preparation of an antigen for complement fixation we advise the use of our strains 34 and 42. This antigen should exhibit generalized and strong fixation properties and prove effective except in the case of the relatively few infections due to certain irregular strains of the gonococcus. With sera giving negative results with this antigen it would seem advisable to conduct a test with an antigen prepared from a combination of irregular strains such as 16, 22, 28, 41, 49, 62 and 77 before giving a negative report. Without doubt at times inconsistencies between the results obtained with this complement fixation test

and clinical and bacteriological findings have been due to the fact that the infecting strain was immunologically unrelated to those used in the antigen. Through the use of these two antigens we believe the chance of this occurring is considerably lessened, but even so no definite assurance may be offered that one or the other would combine with the antibodies produced by all possible variants of the gonococcus.

SUMMARY

1. An analysis by agglutinin absorption methods of the serological relationships of seventy-seven gonococcus strains, isolated from cases of acute and chronic gonorrhea and its complications, indicated that they may *not* be distributed among a number of clear-cut immunological types. These gonococcus strains were representative of those occurring in widely separated geographical localities.

2. Although this investigation has not demonstrated the existence of distinct immunological types to each of which a considerable number of gonococcus strains might be referred, it was found feasible to classify our strains under the three general headings of (*a*) regular, (*b*) intermediate and (*c*) irregular strains.

3. Evidence is submitted which indicates the existence of a marked tendency to antigenic lability on the part of the gonococcus. We believe that instability of antigenic constitution is a general characteristic of the gonococcus group and that the strains exhibiting this tendency may not logically be segregated in one particular type, as has been suggested by Hermanies.

4. Among our regular strains, we have found certain ones which are highly generalized from the antigenic standpoint, and which appear to be representative of a large part of the gonococcus group.

5. Cross absorption experiments have indicated clearly that no definite serological distinction may be drawn between strains isolated from vulvovaginitis cases in children and those from gonorrheal infections in adults.

6. A few representative strains with generalized relationships and good antigenic properties have been designated as suitable

for use in a stock vaccine, and also for the preparation of a polyvalent antiserum.

7. A marked degree of correspondence was noted between strain affinities as revealed by agglutinin absorption and complement fixation tests.

8. Two strains of gonococcus have been selected, each of which covers a large part of the group, and which may be used to advantage in preparing an antigen for complement fixation diagnostic tests. It is also suggested that a second antigen, prepared from a number of selected irregular strains, be employed in conjunction with the one prepared from the two generalized strains.

9. By the use of the simple sterilizable media, which have been described, these strains may be carried in laboratories with limited bactrieological facilities and the antigens prepared quickly, whenever required.

REFERENCES

(1) Torrey, J. C.: Jour. Med. Research, 1907, **16**, 329.
(2) Teague, Oscar and Torrey, J. C.: Jour. Med. Research, 1907, **17**, 223.
(3) Schwartz, H. J. and McNeil, A.: Amer. Jour. Med. Sciences, 1911, **141**, 693.
(4) Huntoon, F. M.: Jour. Infect. Diseases, 1916, **23**, 169.
(5) Perry, M. W. and Kolmer, J. A.: Jour. Immunol., 1918, **3**, 247.
(6) Sands, J. E.: Jour. Immunol., 1920, **5**, 97.
(7) Hooker, S. B.: Jour. Immunol., 1916, **2**, 1.
(8) McClintock and Clark: Jour. Infect. Diseases, 1909, **6**, 127.
(9) Butterfield, C. T. and Neill, M. H.: U. S. Hyg. Lab. Bull., 1920, **124**, 9.
(10) Elser, W. J. and Huntoon, F. M.: Jour. Med. Research, 1909, **20**, 377.
(11) Warren, S. H.: Jour. Path. and Bact., 1921, **24**, 424.
(12) Valentine, Eugenia, and Cooper, Georgia M.: Jour. Immunol., 1919, **4**, 359.
(13) Hermanies, John: Jour. Infect. Diseases, 1921, **28**, 133.
(14) Hermanies, John: Jour. Infect. Diseases, 1921, **29**, 11.
(15) Meinicke, Joffe and Flemming: Zeitschr. f. Hyg., 1906, **52**, 416.
(16) Jötten, K. W.: Munch. med. Wochenschr., 1920, **67**, 1067.
(17) Tulloch, W. J.: Jour. Hyg., 1918, **17**, 317.
(18) Gordon, M. H.: Grt. Britain Med. Research Cmm., Series 3, 1917, 99.
(19) McIntosh and McQueen: Jour. Hyg., 1914, **14**, 409.
(20) Mathers, G. and Herrold, R. D.: Jour. Infect. Diseases, 1918, **22**, 523.
(21) Bemains, T. H. C.: Jour. Path. and Bact., 1920, **23**, 171.
(22) Teague, O., and McWilliams, H. I.: Jour. Immunol., 1917, **2**, 383.
(23) Pearce, Louise: Jour. Exper. Med., 1915, **21**, 289.

(24) Eastwood, Arthur: Jour. Hyg., 1918, **17**, 64.
(25) Griffiths, F.: Jour. Hyg., 1918, **17**, 124.
(26) Scott, W. M.: Jour. Hyg., 1918, **17**, 191.
(27) Walker, E. W. Ainsley: Jour. Hyg., 1918, **17**, 380.
(28) Eberson, F.: Jour. Immunol., 1920, **5**, 345.
(29) Wilson, M. A.: Jour. Immunol., 1920, **5**, 499.
(30) Thomson, David: Lancet, 1918, **195**, 42.
(31) Cook, M. W. and Stafford, D. D.: Jour. Infect. Diseases, 1921, **29**, 561.

STUDIES ON ACUTE RESPIRATORY INFECTIONS

XI. A SEROLOGICAL STUDY OF ALPHA STREPTOCOCCI FROM THE UPPER RESPIRATORY TRACT[1]

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In a previous article of this series there was reported by Doctor Williams (1) and her co-workers a detailed study of the colony formation on standardized blood agar plates of Smith and Brown's (2) alpha type streptococci isolated from the upper respiratory tract. The sugar reactions of these organisms were also given. The results of these studies showed a multiplicity of varieties of streptococci.

In a continuation of this investigation, we have tested the serological relationships of a number of these strains by the agglutination and agglutinin-absorption methods. Very little detailed study on the serological relations of the green producing streptococci seems to have been made and no minute study of the power of colony strains[2] to absorb agglutinins has been reported. Krumwiede and Valentine (3) have reported the results of direct agglutination tests made with members of the streptococcus viridans group obtained from both pathogenic and normal sources. These workers came to the conclusion that types pathogenic for human beings constitute, for all practical purposes, a heterogeneous group.

[1] One of a series of studies carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede. The previous studies in this series were reported in the Journal of Immunology, vi, no. 1, January, 1921, and no. 5, September, 1921. This investigation was made possible by a grant of money from the Metropolitan Life Insurance influenza fund for a part of the expense.

[2] By strain we mean, in this paper, a fishing strain, i.e., the direct descendants of any colony isolated from a plate made from a nasopharyngeal swabbing.

In the following discussion, when we speak of the colony type of strains (I, II, III or IV) or of the sugar fermentation type, we refer to the classification in the previous report of Williams.

SCOPE OF WORK AND DESCRIPTION OF STRAINS

Study of the relationship of alpha streptococci by means of direct agglutination and also absorption of agglutinins, was made with the object of: First, detection of the presence or absence of groups which might indicate an epidemiological relationship to influenza and other respiratory infections. Second, detection of possible correlations between colony, sugar fermentation and agglutination types. Third, comparison of strains from normal, cold and influenza cases. Fourth, making of a detailed study of one case (see case 154 below) to determine the closeness of relationship between the strains from the nasopharynx of the same individual. Fifth, investigation, incidentally, of the more commonly encountered difficulties that limit the value of tests for the differentiation of the particular type of organisms studied.

Work was done on strains from 33 cases classified as follows: 16 influenza patients, 2 doubtful influenza patients, 6 cold cases, 6 normal individuals, 2 measles cases, (cultures from Tunnicliff (4)) and an influenza case studied by Doctor Rosenow (5). Altogether, tests are reported on 112 strains isolated from the above sources.

Serum strains

The sera studied were obtained by immunizing rabbits against each of seven strains.

All seven serum strains except one, showed type I colonies on Smith and Brown blood plates; the exception 140-(1 complex) showed type III colonies The serum used for the greatest number of tests was 154-25.

Case 154 (typical influenza) was the case selected for intensive study. Strain 25 of this case was one of a group of fishings (19 to 25 inclusive) of the mitis type. These fishings gave the

same colony formation on Smith and Brown (2) plates and identical sugar reactions. Planted on chocolate agar, these organisms did not change the color of the medium except that after long cultivation one of the group, strain 154-(21), did show a very faint green. On the Smith and Brown plates they gave small, faint but definite green zones in forty-eight hours at 37°C.; these zones did not change at icebox temperature nor on further incubation at 37°C. These fishings thus approached Smith's gamma type of colony.

This group was further characterized by the homogeneous suspension which resulted when the growth from solid media was rubbed up in 0.8 per cent saline. This was in marked con-

Strains used for production of sera

STRAINS	SOURCE
154-(10) 154-(12)[3] 154-(18) 154-(25) 140-(1 complex)	Nasopharyngeal swabbings from: Typical influenza cases
108-(9)	Doubtful influenza case
67-(2)[3]	Normal individual

trast to the results obtained with some other strains from the same case and from different cases as described later.

The stained films of members of this group after twenty-four hours' growth on chocolate medium, showed Gram-positive, medium-sized cocci with a tendency toward amphophile coloration. The organisms, in contrast to the majority of strains studied in this series, were morphologically comparatively regular. In salt-free phosphate broth[4] they grew in short chains. The length of these chains varied considerably on different occasions; sometimes there were many single organisms or groups of two and three scattered among short chains; at other times

[3] These two sera were produced by Florence Bittman.

[4] Salt-free beef broth containing 0.2 per cent sodium phosphate; reaction, pH 7.8.

there were very few single organisms and the chains were somewhat longer.

Strains from two other cases, namely, 108-(9), 108-(14), 109-(1 and 2) resembled group 154-(19 to 25) in all the particulars (mentioned so far). Strains 100-(2), 100-(3) and 90-(11) were also similar to this group except that they had a tendency toward spontaneous clumping in saline and long chain formation in beef broth.

In contrast to the group (19 to 25) of case 154 all the other colony strains of this case, produced a marked green color on chocolate agar and showed great variability in certain other properties. For instance, in the films of 154-(18) there were often present many irregular organisms which assumed diptheroid shapes. At times these forms were almost absent, at other times they were present in large numbers. Strain 154-(10) also varied in its susceptibility to suspension in saline; sometimes the suspension was quite homogeneous, again it showed a tendency toward spontaneous clumping. Irregular organisms were found occasionally in films of a strain from another case, 152-(6). This strain was also green producing on chocolate agar.

Strain 140-(1 complex)[5] was a fishing from strain 140-(1) (influenza case). On the original Smith and Brown plate, complex colonies of the rosette type were interspersed with a certain number of whetstone colonies. A year ago fishings were made from both types. Plates made from these sub-strains were practically homogeneous as to the type fished. After a year's cultivation on chocolate medium, 140 complex showed both complex and simple colonies. A still later poured plate showed only simple colonies. In our tables we have retained the name 140-(1C) in order to distinguish it from the original mixed strain, and from the sub-fishing 140-(1 simple).

The suspension of 140-(1) in saline was homogeneous, and the film showed Gram-positive cocci growing in chains.

Strain 67-(2) was from a normal individual, a member of the Metropolitan Life Insurance force. The film showed Gram-

[5] Complex refers to Smith and Brown complex types.

positive cocci growing in chains and the suspension in saline was thoroughly homogeneous.

DIRECT AGGLUTINATION

Our sera were obtained by inoculating rabbits with graduated doses of living twenty-four-hour cultures grown on chocolate agar and suspended in 0.8 per cent saline. Nine to twelve doses were given as follows: One dose on each of three consecutive days; then, after an interval of three to four days a second dose and so on. The largest dose was usually about half the growth on a chocolate agar slant in a 6 by 1 inch tube. The sera usually caused complete agglutination through dilutions of 1:600, 1:800 or 1:1000 and partial agglutination at considerably higher dilutions.

Two methods were used in preparing the bacterial suspensions: First, the culture to be tested was transplanted daily on fresh chocolate agar (with some water of condensation) for several days before the test. Finally, an eighteen to twenty-four growth on the chocolate agar was scraped off and carefully rubbed up in 0.7 or 0.8 per cent saline solution. Sometimes, for reasons given below, salt-free beef broth containing 0.2 per cent sodium phosphate was added to the suspension. Effort was made to have the density of the suspension such that, even with some precipitation in the saline control which frequently took place after refrigeration, there remained a definite but not dense cloud in the agglutination tube. Second, when spontaneous or self-agglutination occurred, the growth (as above) was suspended in freshly prepared salt-free phosphate broth instead of salt solution.

The serums, preserved with 0.3 per cent chloroform, were diluted with 0.7 or 0.8 per cent saline. The tests were set up with 0.1 cc. serum dilution and 0.9 cc. bacterial suspension; each strain was set up also with normal rabbit serum and a saline suspension as controls.

The tests were incubated for two hours in a waterbath at 45°C. for the earlier tests; at 50°C. for the later ones. They were then put in the icebox over night and read the following morning.

Difficulties encountered

The greatest difficulty encountered throughout the tests was that of controlling the tendency toward spontaneous agglutination in many strains of the streptococci studied. Most of the strains chosen for the production of serum did not agglutinate spontaneously, but this was far from being the case with many of the other strains tested with the serum produced. The organisms appeared to be extremely capricious. A method that gave fairly satisfactory results at one time was useless at another. An organism which sometimes gave in salt solution a stable homogeneous suspension, at other times would gradually flocculate on further standing. Whether this irregularity was due to slight differences in the suspending media, solutions and manipulations, or to more or less spontaneous variations in the organisms or to other causes was never satisfactorily determined.

To overcome the tendency to spontaneous agglutination, we tried various methods of suspension. On the whole the most satisfactory menstruum for these self-agglutinating organisms was a freshly prepared salt-free phosphate beef broth with a reaction of pH 7.8. Sometimes organisms which had once formed large clumps in saline solution, would remain evenly distributed throughout the phosphate broth after being shaken and allowed to stand over night. At times a reading was found possible with many refractory organisms by growing them over night in the phosphate broth and using the decanted cloudy upper portion for the test. However, the suspension in this portion was often too thin to make a satisfactory reading; at other times when it was heavier, a very slight cloud seemed to persist in the test tubes, even when there was reason to believe that complete agglutination would under other conditions have taken place. For this reason the saline suspension was preferred whenever possible. In certain suspensions it was found advantageous to use a salt solution with the addition of a small portion of broth, just enough of the broth being added to prevent self-agglutination but not enough to interfere with the final reading of the test; one-third phosphate broth was usually sufficient to prevent flocculation in suspensions where this was apt to occur.

Notwithstanding all the difficulties in reading the reactions, after considerable experience with these strains some criteria of differentiation could be established.

It has been said that readings were usually made on the following morning, because the results were much more clear-cut after this interval. With some of the self-agglutinating strains grown in broth, however, just as others had found with self-agglutinating bacteria, a fairly accurate reading could be made after incubation in the water-bath, while on the following morning the controls showed complete agglutination.

The character of the glassware seemed to be an important factor, as the following experiences show. A series of absorption tests was unsuccessful because precipitation took place in all the controls, even with organisms usually giving satisfactory suspensions. These irregularities occurred with one particular batch of saline, which was full of crystals. As other workers reported no irregularities with either the NaCl or distilled water, we concluded that the spontaneous agglutination might have been produced by the formation of an electrolyte from the disintegration of the glass.

Readings

In the tables, complete agglutination with a perfectly clear supernatant fluid is represented by +, while ±, ×, and tr. represent increasing degrees of cloudiness, and – indicates the absence of all agglutination. Frequently sedimentation took place, but after shaking the tubes it was found that there was no macroscopic sign of agglutination. Because of this sedimentation, whenever there was any cause for doubt, the tubes were shaken up after the preliminary reading and the reading often had to be modified in consequence.

On account of the tendency toward spontaneous agglutination of so many of the organisms, it was believed that slight distinctions were important, so that the sign $<$ has been used after a symbol as a modification to indicate that the result was less sharp than the symbol alone would suggest.

Interpretation

In recording our results we have taken as a positive cross-agglutination, a reading that gave a $+$ or $+ <$ at 1:100 and $\pm$, or less, in the normal serum and saline controls.

It is probable that complete agglutination with a highly sensitive antigen at 1:100 is no indication of a closer relationship than partial agglutination at the same dilution with an antigen that flocculates with difficulty. This fact somewhat vitiates general conclusions based on direct agglutination tests with alpha streptococci.

Table 1 shows the results of direct cross-agglutinations with strains of case 154 and of other cases chosen for study on account of their clinical diagnosis or their colony type. It is seen that sera 25 and 18 of case 154 both agglutinate the same strains of the case with the exception of 154-(15 and 16), spontaneous agglutinators and therefore capricious. Another exception is 154-(7) which gives a strong reaction with serum 18 and a weak one with serum 25. In the few tests made with other cases, serum 18 also agglutinates the same strains as serum 25. Serum 12 (case 154) tested only on strains from its own case, agglutinates none of the same strains as sera 25 and 18.

Table 2 sums up the results with the principal sera with which direct agglutinations were carried out. We note that 154-(25), a type I strain from a typical influenza case, produced a serum that gives direct agglutination with about one-third of all of the strains, from one-half of all the cases tested. Strain 140-(1 complex), a type III strain from a typical influenza case, produced a serum that agglutinated about one-eighth of the strains from one-fifth of the cases tested. The serum from 67-(2) a type I strain from a normal throat agglutinated only one strain from a different case, 100-(3) in table 2.

In table 5 are listed the number of cases which have strains tested with each one of the three principal sera and the number of cases which have strains agglutinated by the same sera. These cases are listed acording to their clinical diagnosis. We are justified in concluding that no definite relationship is indicated

between clinical diagnosis and agglutination type if we keep in mind that more influenza than cold and normal cases were tested, especially with sera of strain 154-(25).

Table 4 shows the clinical distribution of those cases in which the sera 154-(25), 140-(10), or 67-(2) agglutinated fully one or more strains from a case, but did not agglutinate all the strains which were tested from that case. It can be seen that although 18 cases had one or more strains agglutinated by one of the three test sera, 11 of these cases had other strains that were not agglutinated by these same sera, thus giving evidence of a multiplicity of agglutinative types.

Table 2 shows us that the influenza case 154, 22 strains of which were tested with sera 154-(25), 154-(18) and 154-(12), and the influenza case 140, 8 fishings of which were tested with serum 140-(1c) each have at least two entirely distinct agglutination types, while the normal case 67, of which 4 fishings were tested with serum 67-(2), has only one type. As far as comparisons can be drawn from so meagre a number of strains and cases, these results together with those just cited from table 4, correspond with those obtained from the cultural study of strains in this series of cases; that is, although the strains isolated from cold and influenza patients may be of several types, those from normal subjects are more apt to be all of one type.

On the basis of colony type no clear-cut relationship is indicated in table 1. Serum 154-(25) (type I) agglutinates some strains of type I, II, and III and a doubtful type IV, though more of type I. This is to be expected, as the type I strains were found to be far more numerous and were present in a greater number of cases. Serum 140-(1c) (type III) agglutinates several types also. The one strain from a different case (100) agglutinated by serum 67-(2) (type I) is likewise of type I. It is a strain that was also agglutinated by serum 154-(25).

It is important to note that the sugar types in this series do not seem to run parallel to the serological types. However, it is a striking fact that with all the strains previously discussed, which resemble somewhat the gamma type, namely 154-(19) to (25), 108-(9), 108-(14), 109-(1 and 2), 100-(2), 100-(3), 90-(11), the sugar reactions are identical.

TABLE 1

Direct agglutination of alpha streptococci with monovalent rabbit sera

CASE	STRAIN	DIAGNOSIS	COLONY TYPE	SUGAR TYPE	SERUM 154–25					CONTROLS		SERUM 154–18					CONTROLS		SERUM 154–12					CONTROLS	
					100	200	400	600	800	Normal serum	Saline	100	200	400	600	800	Normal serum	Saline	100	200	400	600	800	Normal serum	Saline
154	1	Influenza	I	Saliv.	+	+	+	+	+	+	+								+	+	+			+	+
+ ±	2	Influenza	I	Saliv.	+	+	+	+	+	±	+								+	+	+			+	+
	4	Influenza	I	Saliv.	±	±	±	×	×	—	×	×	×	×	×	×	tr.	tr.	±	±	±			tr.	tr.
	5	Influenza	I	Ignavus	+	+	+	+	+	+	+	×	×	×	×	×	—	×	+	+	+			±	±
	6	Influenza	I	Ignavus	—	—	—	—	—	—	—	×	tr.	tr.	tr.	tr.	tr.	—	+	+	+			tr.	tr.
	7	Influenza	I	Saliv.	±	±	×	×	tr.	—	—	+	+	+	+	×	—	—	tr.	tr.	tr.			tr	—
	8	Influenza	I	Saliva	+	±	±	±	×	—	—	+	+	+	+	±	tr.	—	×	×	×			tr.	tr.
	10	Influenza	I	Saliv.	+	+	+	+	+	—	—	+	+	+	+	+	×	×	±	±	×			tr.	tr.
	11	Influenza	I	Ignavus	+	+	+	+	+	+	+	+	±	±	±	±	×	±	+	+	±			±	±
	12	Influenza	I	Ignavus	±	±	±	±	±	±	±	±	±	±	±	±	×	±	+	+	+			±	±
	13	Influenza	I	Saliv.	+	±	×	tr.		tr.	—	+	+	±	×	tr.	—	—	×	×	×			—	—
	14	Influenza	I	Saliv.	+	+	+	+	+	+	+	+	+	+	+	+		+	×	×	×			tr.	tr.
	15	Influenza	I	Saliv.	+	±	±	±		±	±	—	—	—	—	—	—	—	—	—	—			—	—
	16	Influenza	I	Saliv.	±	×	tr.	tr.	tr.	tr.	—	+	+	+	+	+	—	—	×	×	×			×	×
	17	Influenza	I	Saliv.	+	+	+	±	±	—	—	+	+	+	+	+	±	—	—	—	—			—	—
	18	Influenza	I	Saliv.	+	+	+	+		tr.	—	+	+	+	+	×	—	—	—	—	—			—	—
	19	Influenza	I	Mitis	+	+	+	+		—	—	+	+	+	±	±	—	—	tr.	tr.	tr.			—	—
	20	Influenza	I	Mitis	+	+	+	+		tr.	×	+	+	+	×	tr.	—	—	×	×	×			×	+
	21	Influenza	I	Mitis	+	+	+	±		—	tr.	+	+	+	×	—	—	—	—	—	—			—	—
	22	Influenza	I	Mitis	+	+	+	±		—	—	+	+	+	±	±	—	—	×	±	±			×	±
	24	Influenza	I	Mitis	+	+	±	±		—	—	+	+	+	±	tr.	—	—	—	—	—			—	—
	25	Influenza	I	Mitis	+	+	+	+		—	—	+	+	+	×	tr.	—	—							
130	5	Influenza	I	Saliv.	+	+	+	+		—	—														
	6	Influenza	I	Saliv.	+	+	+	+		—	—														
	12IV	Influenza	IV	Saliv.	—	—	—	—		—	—														
151	3A	Influenza	I	Saliv.	+	+	±	×	×	tr.	—														

152	5	Influenza	I	Saliv.	+<	±	±	±	±	−	−													
	6	Influenza	I	Saliv.	+	+<	±	±	±	−	−	+	+	+	±	±	×	tr						
157	1B	Influenza	I or IV	Saliv.	+	+	±	tr.	tr.	−	−													
	3B	Influenza	I or II	Mitis (2)	+	+	+	±	±	−	−	+	+	+	+	×	tr.	−						
	6	Influenza	IV	Saliv.	−	−	−	−	−	−	−													
												SERUM 140-1c					CONTROLS		SERUM 67-2					CONTROLS
161	2	Influenza	III	Saliv.	×	tr.	tr.		−	−	−	±	×	×		tr.	−	−						
	5	Influenza	III	Mitis	×	×	tr.		tr.	−	−	tr.	tr.	tr.		tr.	tr.	tr.						
	18	Influenza	III	Non-Hemol. II	−	−	−		−	−	−	−	−	−		−	−	−						
	18 like 90	Influenza	I	Equinus	±	×	×		×	×	×	tr.	tr.	tr.		tr.	tr.	tr						
	19	Influenza	III	Mitis	×	−	−		−	−	−	−	−	−		−	−	−						
161[2]	6	Influenza	III	Mitis	−	−	−		−	−	−	−	−	−		−	−	−						
	9A	Influenza	III	Saliv.	×	×	tr.		tr.	tr.	tr.	tr.	tr.	tr.		tr.	tr.	tr.						
	16B	Influenza	III	Mitis	−	−	−		−	−	−	−	−	−		−	−	−						
165	4	Influenza	I	Mitis	−	−	−		−	−	−	×	×	×		×	×	×	+	+	+		+	+
	5	Influenza	I	Saliv.	×	×	tr.	tr.	tr.	−	−	−	−	−		−	−	−	−	−	−		−	−
	7	Influenza	I	Saliv.	tr.	tr.	tr.	tr.	tr.	−	−	+<	±	tr.		−	−	−						
	12	Influenza	III	Saliv.	−	−	−		−	−	−	−	−	−		−	−	−						
128	5	Influenza	I	Mitis	±	×	×	×	×	×	×													
147	3	Influenza	I	Saliv.															+	tr.	−	−	−	−
	17	Influenza	IV	Mitis	−	−	−	−	−	−	−													
	2	Influenza	IV	Mitis	−	−	−	−	−	−	−													
	23	Influenza	IV	Mitis	−	−	−	−	−	−	−													
140	1C	Influenza	III	Mitis	−	−	−	−	−	−	−	+	+	+	+	+	−	−						
	1S	Influenza	I	Mitis								+	+	+	+	+	−	−						
	1S	Influenza	I or III	Mitis								+	+	+	+	+	−	−						
	4	Influenza	I	Ignav.								+	+	+	+	+	−	tr.						
	526	Influenza	I	Ignav.								−	−	−	−	−	−	−						
	7	Influenza	II	Saliv.								−	−	−	−	−	tr.	−						
	8	Influenza	I	Saliv.								−	−	−	−	−	−	−						
158	18	Influenza	III	Mitis	tr.	tr.	tr.		tr.	−	−	tr.	tr.	tr.	tr.	tr.	−	−						
159	14	Influenza	III	Saliv.	+	+	+		+	+	+	+	+	+		+	+	+						
	15	Influenza	III	Mitis	−	−	−		−	−	−	−	−	−		−	−	−						
132	1	Influenza	II	Mitis	+	+	+	+	+	tr.	tr.													
	3	Influenza	II	Saliv.	±	×	×	×	tr.	×	tr.													
	14	Influenza	II	Mitis	+	+	×	×	×	−	−													

TABLE 1—*Continued*

CASE	STRAIN	DIAGNOSIS	COLONY TYPE	SUGAR TYPE	SERUM 154-25					CONTROLS		SERUM 154-18					CONTROLS		SERUM 154-12					CONTROLS	
					100	200	400	600	800	Normal serum	Saline	100	200	400	600	800	Normal serum	Saline	100	200	400	600	800	Normal serum	Saline
168[3]	10	Influenza	II	Saliv.	±	×	×	×	×	×	×														
	11B	Influenza	II	Mitis	×	×	×	×	×	×	×														
	12A	Influenza	II	Mitis	−	−	−	−	−	−	−														
169[2]	2B	Influenza	IV	Mitis	−	−	−	−	−	−	−														
	3	Influenza	IV	Mitis	−	−	−	−	−	−	−														
	*6	Influenza	IV		−	−	−	−	−	−	−														
	8	Influenza	IV	Mitis	−	−	−	−	−	−	−														
146	1 and 2	Influenza	IV	Saliv.	−	−	−	−	−	−	−														
	3[2]	Influenza	IV	Saliv.	+	+	+	+	+	×	+														
	6[2]	Influenza	IV	Ignav.	−	−	−	−	−	−	−														
Rosenow	A[2]	Influenza			−	−	−	−	−	−	−														
	2800	Influenza			−	−	−	−	−	−	−														
108	9	Doubtful influenza	I	Mitis	+	+	+	+	+	−	−								±	×	−		−		−
	14	Doubtful influenza	I	Mitis	+	+	+	±	×	−	−														
100	2	Doubtful influenza	I	Mitis	+	+	+		+	−	−														
	3	Doubtful influenza	I	Mitis	+	+	+		+	tr.	tr.								+	±	×		−		−
109	1 and 2	Cold	I	Mitis	+	+	+	+	+	−	−														
	15	Cold	I	Mitis or saliv.	−	−	−	−	−	−	−	+	+	±		−	−	−							
170	3A	Cold	I or III	Mitis	−	−	−	−	−	−	−	+	+	±	×	×	−	−	−	−	−	−	−		−
	5A	Cold			+	+	+	+	+	×	tr.														
	5B	Cold			+	+<		×	tr.	tr.	tr.														
	6B	Cold	I	Saliv.	±	×	×	×	×	−	tr.	×	×	×	×	×	×	×	×	×	×	×	×		tr.
	7B	Cold	I	Mitis	+	±	±	±	±	tr.	×	×	×	×	×	×	×	×	±	±	×	×	×		×
32	3	Cold	I	Saliv.	+	+	×	×	×	tr.	tr.	×	×	×	×	×	×	×	±	±	×	×	×		×
	4	Cold	I	Mitis	×	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−
	6	Cold	I	Mitis	+	×	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−
127	1	Cold	I	Mitis or saliv.	−	−	−	−	−	−	−	tr.	−	−	−	−	−	−	−	−	−	−	−		−

	8	Cold	I	Mitis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
	10	Cold	I	Saliv.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
21	1	Cold	I	Mitis	+	+	+	+<	+<	—	—	tr.	tr.	tr.	tr.		—	—	±	×	—	—	—		—
	3	Cold	I	Fecalis	+	+	+	±	±	—	—	tr.	—	—	—		—	—	×	—	—	—	—		—
69	1	Cold	II	Mitis	±	×	×	tr.	tr.	—	—	—	—	—	—		—	—	—	—	—	—	—		—
	2	Cold	II	Mitis	+	+	×	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—		—
90	11	Normal	I	Mitis	+	+	+	+	+	+	+														
67	1	Normal	I	Mitis	—	—	—	—	—	—	—	—	—	—	—		—	—	+	+	+	+	+<		—
	2	Normal	I	Mitis	—	—	—	—	—	—	—								+	+	+	+	+		—
	11	Normal	I	Mitis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+<	+<		—
33[2]	2	Normal	I and II	Equinus	+	+	±	×	×	—	—	tr.	—	—	—	—	—	—	tr.	—	—	—	—		—
	5	Normal	I		+	+	×	tr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
	8	Normal	I		+	+	+	+<	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
43	1	Normal	I and III	Saliv.	—	—	—	—	—	—	—	+	+	—	—	—	tr.	—	—	—	—	—	—		—
	2	Normal	II	Saliv.	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—		—
	5	Normal	I and III		—	—	—	—	—	—	—	+	+	+	±	—	—	—	—	—	—	—	—		—
78[2]	1	Normal	I	Mitis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
	4	Normal	II		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
	6	Normal	I	Mitis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—
79[2]	1	Normal	I	Saliv.	+	+	+	±	±	tr.	×	±	±	×	×		tr.	×	×	×	×	×	×		×
	4	Normal	I	Saliv.	×	tr.	tr.	tr.	tr.	—	—	×	tr.	tr.	tr.	tr.	tr.	—	×	—	—	—	—		—
J. S.		Measles			+	+	+	+	+	—	—														
Stable		Measles										tr.	tr.	tr.		tr.	tr.	tr.							

* Strain 169^2–6 was bile soluble.

Note: The following tests have been omitted from this table: Serum 154–18 tested with strain 103–9 agglutinated the strain through a dilution of 1–200. Serum 140–1C tested with strains 154–25, 151–3A, 152–5, 152–6, 157–3B agglutinated none of these strains. Serum 67-2 tested with strains 154-13, 154-25, 130-5, 151-3A, 152-5, 152-6, 157-3B agglutinated none of these strains.

TABLE 2

Summary of results of direct agglutination tests with sera 154–25, 140–1C, 67–2

SERUM	NUMBER OF STRAINS TESTED		NUMBER OF STRAINS SHOWING POSITIVE AGGLUTINATION AT 1:100			NUMBER OF CASES TESTED	NUMBER OF CASES HAVING ANY STRAINS AGGLUTINATED AT 1:100	
	Total number	Number not from case of serum strain	Total number	Number not from case of serum strain				
				number	*per cent*		*number*	*per cent*
154–25	103	82	39	26	31.7	32	15	46.9
140–1C	58	50	10	6	12.0	20	4	20.0
67–2	40	36	5	1	2.7	18	1	5.5

TABLE 3

Clinical distribution of those cases having one or more strains agglutinated at 1:100 by sera 154–25, 140–1C, or 67–2

SERUM	INFLUENZA				COLDS		NORMALS		MEASLES	
	Number of cases tested		Cases having agglutinating strains		Number of cases tested	Cases having agglutinating strains	Number of cases tested	Cases having agglutinating strains	Number of cases tested	Cases having agglutinating strains
154–25	16	2 D.I.*	5	2 D.I.*	6	4	6	3	1	1
140–1C	8		1		6	2	5	1	1	0
67–2	8	1 D.I.	1		5	0	5	0	0	0

* D.I. = Doubtful influenza.

TABLE 4

Showing the number of cases in which a multiplicity of agglutination types was found among the strains of the same case

SERUM	NUMBER OF CASES TESTED AS TO SEVERAL STRAINS, HAVING ONE OR MORE AGGLUTINATING STRAINS				NUMBER OF CASES IN WHICH ALL STRAINS TESTED WERE AGGLUTINATED				NUMBER OF CASES IN WHICH SOME BUT NOT ALL STRAINS TESTED WERE AGGLUTINATED			
	Influenza		Colds	Normals	Influenza		Colds	Normals	Influenza		Colds	Normals
154–25	5	2 D.I.*	4	2	2	1 D.I.*	1	1	3	1 D.I.*	3	1
140–1C	2		1	1	0		0	1	2		1	0
67–2	0		0	1†	0		0	0	0		0	0
Total	7	2	5	4	2	1	1	2	5	1	4	1

* D.I. = Doubtful influenza.

† Serum strain.

TABLE 5

Agglutinin absorption tests with sera of rabbits inoculated with strain 154-25

DIRECT AGGLUTINATION							AGGLUTINATING STRAIN	AGGLUTINATION BY ABSORBED SERUM					
1:50	1:100	1:200	1:400	1:800	Normal serum	Saline		1:50	1:100	1:200	1:400	1:800	Absorbing strain
							(a) Rabbit c						
+	+	+	+	+	−	−	154-25	−	−	−	−	−	154-25
+	+	+	+	+	−	−	154-19	−	−	−	−	−	154-19
+	+	+	+	+	−	−	154-21	−	−	−	−	−	154-21
+	+	+	+	+	tr.	tr.	154-10	±	±	×	×	tr.	154-10
+	+	+	+	±	−	−	154-18						
							154-19	−	−	−	−	−	154-25
							154-25	−	−	−	−	−	154-19
							154-21	−	−	−	−	−	154-25
							154-25	−	−	−	−	−	154-21
							154-10	+<	+<	±	±	tr.	154-25
							154-25	+	×	−	−	−	154-10
							154-18	−	−	−	−	−	154-10
							(b) Rabbit c						
+	+	+	+	+	−	−	154-25	−	−	−	−	−	154-25
+	+	+	+	×	−	tr.	157-3B	×	×	tr.	−	−	157-3B
+	+	+	+	+<	−	−	108-9	−	−	−	−	−	108-9
+	+	+	+	+	tr.	tr.	100-3	tr.	tr.	tr.	tr.		100-3
							157-3B	×	×	−	−	−	154-25
							154-25	+	+	+	±	−	157-3B
							108-9	−	−	−	−	−	154-25
							154-25	+	+	±	−	−	108-9
							100-3	×	tr.	tr.	tr.		154-25
							154-25	+	+	±	−		100-3
							(c) Rabbit c						
+	+	+	+	+	−	−	154-25	tr.	−	−	−	−	154-25
+	+	+	+	+	−	−	109-1 and 2	tr.		−	−	−	109-1 and 2
+	+	+	+	±	−	−	152-6	−	−	−	−	−	152-6
+	+	+	+<	×	−	−	157-3B						
+	+	+	+	+	−	−	154-10						
							109-1 and 2	×	−	−	−	−	154-25
							154-25	+	+	±	−		109-1 and 2
							152-6	tr.	tr.	−	−	−	154-25
							154-25	+	+	×	tr.	−	152-6
							109-1 and 2	+<	±	tr.	−	−	152-6
							157-3B	tr.	tr.	−	−	−	152-6
							154-10	+	+	+	+	±	152-6
							(d) Rabbit a						
+	+	+	+	+<	−	−	154-25	tr.	−	−	−	−	154-25
+	+	+	+	+<	−	−	154-18	−	−	−	−	−	154-18
							154-18	−	−	−	−	−	154-25
							154-25	+	+	×	tr.	−	154-18

TABLE 5—*Continued*

DIRECT AGGLUTINATION							AGGLUTINATING STRAIN	AGGLUTINATION BY ABSORBED SERUM					
1:100	1:200	1:400	1:600	1:800	Normal serum	Saline		1:100	1:200	1:400	1:600	1:800	Absorbing strain
(e) Rabbit a													
+	+	+	+	+	−	−	154-25	−	−	−	−	−	154-25
+	+	+	+	+	×	−	130-5	tr.	tr.	−	−	−	130-5
							130-5	tr.	−	−	−	−	154-25
							154-25	±	±	×	tr.	−	130-5
(f) Rabbit a													
+	+	+	+	+	−	tr.	154-25	−	−	−	−	−	154-25
+	+	+	+	+	−	−	J. S.	−	−	−	−	−	J. S.
							J. S.	−	−	−	−	−	154-25
							154-25	+	+	+	+	+	J. S.

Discussion

There seem to be related agglutinins present among a number of strains of alpha streptococci. These agglutinins must be present in considerable quantity, since no account has been taken of any reactions but those complete at 1:100 dilution. It is possible that with sera produced against a sufficient number of strains, a classification of all alpha streptococci according to agglutination type might be possible.

The presence of an epidemic strain among the organisms isolated from influenza cases, is not indicated for several reasons. In the first place not enough strains were agglutinated by any one of the five sera tested to suggest that any of the serum strains represented an epidemic strain. However, a sufficiently marked relationship was present to rule out the possibility that the serum strains chosen were chance invaders which happened to persist in the presence of an epidemic strain.

In the second place, the strains showing relationship with the serum strains, were distributed among all the clinical types studied, that is, influenza, measles, colds and normal. Two influenza sera agglutinated a number of normal strains and, therefore, sera against these normals might have agglutinated

influenza strains which makes less significant the lack of relationship between the normal strain 67-(2) and all other strains tested with the exception of one, 100-(3). Nevertheless, it is worth noting that Krumwiede and Valentine found that several strains from normal tonsils were agglutinated by sera produced by pathological strains, while the sera produced by these tonsillar strains did not agglutinate pathological strains. Krumwiede and Valentine attributed this phenomenon partly to the greater tendency of the normal strains towards spontaneous agglutination. Although many of our pathological strains were self-agglutinating, it is a fact that out of the four normal cases which had strains agglutinated by our two influenza sera, three cases had only self-agglutinating strains; however, in the tests recorded on these strains, it was possible to control the antigen successfully. On the whole, there may be a real significance in the absence of cross-agglutination by the normal serum.

In the third place, both influenza cases, that is, 154 and 140 showed the presence of at least two serologically unrelated alpha streptococci. Some, but not all of the strains from several other influenza cases, when tested, agglutinated with the same serum.

ABSORPTION OF AGGLUTININS

Absorption tests were carried out with a number of different strains which had showed marked direct agglutination reactions. Besides the five sera recorded in the direct agglutination tables, two additional sera were used, one from strain 154-(10) and one from strain 108-(9); the latter as previously stated, was very similar to 154-(25) in a number of biological characteristics, as well as in its high direct agglutination by serum 154-(25). Case 108 had been diagnosed as one of doubtful influenza.

Method

With influenza strains grown on solid media it was difficult to get a sufficient number of packed cells from young growths. The organisms were, therefore, grown overnight at 37°C. in

bottles containing 200 cc. of salt-free phosphate beef broth. The broth cultures were centrifuged at high speed in 50-cc. containers. The organisms were then transferred to a graduated tube and again centrifuged at high speed after which the supernatant fluid was decanted; the right amount of serum was added to the packed cells to permit complete absorption to occur with the homologous strain whenever possible. Saline was now added to give a serum dilution of 1:10 when the serum titer was 1:800 or over, and of 1:5 when the titer was 1:600. The suspension was then incubated for three hours with frequent shaking at 50°C. and put in the icebox over night.

The following day, agglutination tests were carried out with the centrifuged supernatant liquid. In adding the serum to the packed cells, we calculated as Valentine and Cooper (6) had suggested, that one half the amount of the reading might fairly represent dried organisms.

Difficulties encountered

In carrying out the tests it was frequently difficult to get complete absorption in the controls. This may have been due to technical shortcomings, or to the self-agglutinating nature of the antigen. The commercial centrifuge tubes are often carelessly graduated so that the readings on the packed cells and on the saline added are inaccurate and, therefore, not comparable in the different tubes. When the quantities are small the error may be significant.

One of the most difficult factors to control is the varying sensitivity of different antigens. For instance, a certain amount of antigen A may absorb all the agglutinins of the homologous serum; the same amount of antigen B may leave unbound agglutinins which in the subsequent agglutination test, combine with B. Of course it is possible in such a case that serum A has more agglutinins for B than for its homologous strain, but from our tests it seems far more likely that the result is due to the greater sensitivity of antigen B to all flocculating agents, bringing about an agglutination in the presence of a very few unbound agglu-

tinins which would be insufficient to affect A. It is only by a very careful comparison of controls and by cross-absorptions, that a correct interpretation of such contrasting results is possible. As was pointed out by Valentine and Cooper (6), the danger of non-specific absorption with too large a volume of packed cells is not great. We made the reverse test by treating our packed cells with increasing doses of serum and found that our largest and our smallest serum doses gave similar readings well within the limits of error.

Results

In table 5, a, b, c, d, e, and f represent six different sets of absorptions with serum 154-(25) obtained from two different rabbits. Some of the tests with serum of rabbit A were among the first carried out; the lowest dilution was 1:100. Afterwards, it was found better to make the lowest dilution 1:50 if the serum had a titer of 1:800 or more, and 1:25 if it had a titer of 1:600. Strains 154-(10), 154-(18), 152-(6), 157-(3B), 130-(5), J. S., (measles) were all green producing on chocolate agar. Strains 154-(19 to 25) 108-(9), 109-(1 and 2) and 100-(3), as has been previously stated, did not produce green on chocolate agar.

The following tests were recorded on each strain tested, for example, "strain x":

1. Agglutination by unabsorbed serum.
2. Agglutination by serum absorbed by "strain x."
3. Agglutination by serum absorbed by a serum strain.
4. Agglutination of the serum strain by serum which had been absorbed by "strain x."

The following facts were noted: With all but one of the strains tested there was some absorption of agglutinins shown by the fact that the titer of the absorbed serum when tested with its homologous strain, was greatly reduced. This reduction could not have been due merely to mass absorption, for serum 154-(25) when absorbed by a non-agglutinating strain and then tested with strain 154-(25), gave complete agglutination in a dilution

of 1:600 and a marked reaction in 1:1000. Therefore, the absorption was probably specific. When entire mutual absorption was used as a criterion, identity seemed to be complete for the two strains, 154-(19) and 154-(21) belonging to the same group as 154-(25) (table 5, a). Tests on other strains of case 154, namely, (20), (22) and (24) (not recorded), gave similar results. Serum 154-(25) absorbed by 154-(10) and 154-(18) which were green producers giving a full direct cross-agglutination, brought 154-(18) down completely through a dilution of 1:100, and 154-(10) through a dilution of 1:50 (table 5, a and d). This serum absorbed by strains from other cases, similar to 154-(25) in not producing green on chocolate agar, namely, 108-(9), 100-(3) and 109-(1 and 2), agglutinated these strains almost completely at a dilution of 1:200, (tables 5, b and c). With cross-agglutinating strains from other cases producing green on chocolate agar we obtained a gradation in the results. J. S., a measles strain, though completely agglutinated by serum 154-(25) did not absorb any agglutinins from this serum. Serum 154-(25) agglutinated its homologous strain almost completely through 1:400 after absorption by 157-(3B). Absorption of this serum by strain 152-(6) left agglutinins to about the same extent as absorption by 154-(18), while absorption by 130-(5) removed most of the agglutinins (table 5, f, b and c).

We observed further, that although absorption of serum 154-(25) by 154-(10) removed the agglutinins from 154-(18), absorption by 152-(6) removed them scarcely at all for 154-(10), only partially for 109-(1 and 2) and almost completely for 157-(3B).

Another important point is that although this serum 154-(25) when absorbed by strain 154-(10), lost most of the agglutinins for the homologous strain except in the lowest dilution, it lost them only partially for the absorbing strain, and that the serum, fully absorbed by its homologous strain, 154-(25), retained sufficient agglutinins to cause almost complete flocculation of strain 154-(10) (table 5, a). This is another illustration of the greater sensitivity of some of our antigens to traces of agglutinins in a serum, or in fact to any agency that favors flocculation.

The results with serum 108-(9) are given in table 6. We see

that both strain 154-(25) and strain 152-(6) are agglutinated as completely as the homologous strain. We also observe a very pretty grading in their capacity for absorbing the specific agglutinins of the serum strain. Absorption is practically complete with one strain of the same case, 108-(14); with 154-(25), simi-

TABLE 6

Agglutinin absorption tests with serum 108-9 (rabbit h)

DIRECT AGGLUTINATION								AGGLUTINATING STRAIN	AGGLUTINATION BY ABSORBED SERUM						
1:50	1:100	1:200	1:400	1:600	1:800	Normal serum	Saline		1:50	1:100	1:200	1:400	1:600	1:800	Absorbing strain
+	+	+	+	+<	+<	–	–	108-9	–	–	–	–	–	–	108-9
+	+	+	+	+	+	–	–	108-14	tr.	–	–	–	–	–	108-14
+	+	+	+	+<	+<	–	–	154-25	tr.	–	–	–	–	–	154-25
+	+	+	+	+<	+<	–	–	152-6	–	–	–	–	–	–	152-6
								108-14	tr.	–	–	–	–	–	108-9
								108-9	tr.	–	–	–	–	–	108-14
								154-25	–	–	–	–	–	–	108-9
								108-9	±	–	–	–	–	–	154-25
								152-6	tr.	–	–	–	–	–	108-9
								108-9	+	±	–	–	–	–	152-6

lar in several respects, it is less complete; while with 152-(6), more markedly green-producing, there is no absorption apparent in the lowest dilution of the absorbed serum, but complete absorption from 1:200 upwards.

Table 7 records a set of absorptions of serum 140-(1 complex).[6] In this table is recorded the only instance which we have observed of a practically complete absorption of a serum by a strain from another case. The organism in question 170-(3A) had a slight tendency toward spontaneous clumping. It may be worth while to quote from our notes on this test:

Strain 170-(3A) was grown in phosphate broth. After incubation and a night in the refrigerator, there was almost complete precipitation in all the tubes with a very faint haze in the controls. After shaking,

[6] When this serum was used for absorption tests it was already several months old and was greatly reduced in titer.

neither the serum nor broth control showed any vestige of agglutination, nor did any of the tubes containing absorbed serum; but agglutination was marked with the unabsorbed serum, so that the reading was absolutely clear-cut.

We observed that this serum absorbed by 170-(3A) and cross-agglutinated with strain 140-(1 complex), showed a trace of agglutination in the lowest dilution tested. Because of the deterioration of the serum and of the fact that we had no serum produced by strain 170-(3) with which to control our results,

TABLE 7

Agglutinin absorption tests with serum 140-1 complex (rabbit e)

DIRECT AGGLUTINATION							AGGLUTINATING STRAIN	AGGLUTINATION BY ABSORBED SERUM					
1:25	1:50	1:100	1:200	1:400	Normal serum	Saline		1:25	1:50	1:100	1:200	1:400	Absorbing strain
+	+	+	+	+	tr.	–	140-1C	×	×	tr.	–	–	140-1C
+	+	+	+	+<	–	–	140-4	–	–	–	–	–	140-4
							140-4	–	–	–	–	–	140-1C
							140-1C	±	×	tr.	–	–	140-4
+	+	+	+	+<	–	–	140-1C	–	–	–	–	–	140-1C
+	+	+	+	+	–	–	170-3A	–	–	–	–	–	170-3A
							170-3A	–	–	–	–	–	140-1C
							140-1C	tr.	–	–	–	–	170-3A

we do not attach great importance to this single case of identity of two strains from distinct sources. It is interesting to note, however, that 170-(3A) shows a tendency to produce type III colonies on a Smith and Brown plate, thus resembling strain 140-(1 complex).

Comparison of strains from the same case

We stated at the beginning that one part of our problem was the detailed study of a single case. This is of value because the degree of relationship among cross-agglutinating strains of the same case, judged by the criterion of absorption, helps us to interpret results with strains isolated from different cases. Such

study is of assistance in distinguishing between an organism that tends to preserve fixed biological characters and one that is constantly subject to slight modifications.

We chose for this study case 154 because it was a well marked case of influenza and all of the fishings were of the same colony type on blood agar plates. We found that the group 154-(19 to 25) absorbed completely the agglutinins of serum 154-(25). Absorptions of serum 154-(25) by strains 154-(18) and 154-(10) were incomplete. To make further tests we also obtained sera from strains 154-(18) and 154-(10). The full results of our various absorption tests are recorded in table 8. The results are somewhat difficult to interpret because the strains represent antigens of such varying sensitivity. All three strains are undoubtedly very closely related.

For instance, as previously noted, although strain 154-(25) absorbs all of its own agglutinins from the homologous serum, the serum so absorbed remains able to agglutinate the more sensitive strain 154-(10); that is, the same volume of packed cells which is sufficient to absorb all its own agglutinins from a certain serum dose when we use cells of strain 154-(25), is inadequate when we use packed cells of strain 154-(10), to achieve the same result. If, however, the serum did not contain agglutinins in larger number or more specific for 154-(25) than for 154-(10) the same volume of cells of the latter strain should remove all agglutinins from serum 154-(25). Since it does not do this, we conclude that strains 154-(25) and 154-(10) are very similar but not identical.

The serum 154-(10) at first suggests a clear-cut interpretation. Strain 154-(25) shows a low direct agglutination with serum 10; strain 154-(10) absorbs all the agglutinins for strain 154-(25) and strain 154-(25) only partially absorbs them for strain 154-(10). But our conclusions are modified when we observe in table 9 that strain 154-(10) has a higher titer with serum 154-(25) than the homologous strain, and also higher than it has with its own serum. We might, therefore, expect that serum 154-(10) would agglutinate strain 154-(25) in low dilution and that its agglutinins would all be absorbed by strain 154-(10). On the

TABLE 8

Agglutinin absorption tests with sera 154-25, 154-10, 154-18

DIRECT AGGLUTINATION									AGGLUTINATING STRAIN	AGGLUTINATION BY ABSORBED SERUM							
1:25	1:50	1:100	1:200	1:400	1:600	1:800	Normal serum	Saline		1:25	1:50	1:100	1:200	1:400	1:600	1:800	Absorbing strain
									Serum 154-25 (rabbit C)								
	+	+	+	+		+	—	—	154-25		—	—	—	—		—	154-25
	+	+	+	+		+	tr.	tr.	154-10		±	±	×	×		tr.	154-10
									154-10		+<	+<	±	±		tr.	154-25
									154-25		+	×	—	—		—	154-10
									Serum 154-10 (rabbit J)								
	+	+	+	+		+	tr.	tr.	154-10		×	×	×	tr.		tr.	154-10
	+	+	±	tr.		—	—	—	154-25		—	—	—	—		—	154-25
									154-25		—	—	—	—		—	154-10
									154-10		+	+	×	×		×	154-25
									Serum 154-25 (rabbit C)								
	+	+	+	+		+	—	—	154-25		±	×	—	—		—	154-25
	+	+	+	+		+	—	—	154-18		—	—	—	—		—	154-18
									154-18		±	×	tr.	—		—	154-25
									154-25		+	+	tr.	—		—	154-18
									Serum 154-18 (rabbit D)								
+	+	+	+		+<		—	—	154-18	×	×	—	—		—		154-18
+	+	+	±		—		—	—	154-25	×	×	—	—		—		154-25
									154-25	—	—	—	—		—		154-18
									154-18	+	+	×	tr.		tr.		154-25
									Serum 154-10 (rabbit J)								
+	+	+	+	+			×	tr.	154-10	±	×	×	tr.	tr.			154-10
+	+	+	+	±			—	—	154-18	—	—	—	—	—			154-18
									154-18	—	—	—	—	—			154-10
									154-10	+	±	tr.	tr.	tr.			154-18
									Serum 154-18 (rabbit D)								
+	+	+	+		±		—	—	154-18	—	—	—	—		—		154-18
+	+	+	+		+		—	—	154-10	—	—	—	—		—		154-10
									154-10	+	+	+	±		×		154-18
									154-18	±	×	×	tr.		—		154-10

TABLE 9

Agglutination of strain 154-10 by sera 154-10, 154-25, 154-18

SERUM	SERUM DILUTIONS						CONTROLS		SERUM TITER FOR HOMOLOGOUS STRAIN
	1:600	1:800	1:1000	1:1200	1:1400	1:1600	Normal serum	Saline	
154-10	+	+	+	±	±	±	×	tr.	1-1000
154-25	+	+	+	+	+	+	×	tr.	1-1000
154-18	+	+	+	+	+	+	×	tr.	1-600

TABLE 10

Summary of agglutinin absorption tests

CASE	NUMBER OF STRAINS TESTED SHOWING COMPLETE AGGUTINATION AT 1:100	NUMBER OF STRAINS TESTED FOR AGGLUTININ ABSORPTION	STRAINS TABULATED ACCORDING TO THE DEGREE OF AGGLUTINATION OF HOMOLOGOUS STRAIN BY SERUM ABSORBED BY INDICATED STRAINS							SERUM
			Agglutination less than × at lowest dilution*	Agglutination ± or × at lowest dilution*	Complete agglutination at 1:50	Complete agglutination at 1:100 less than ± at higher dilutions	Complete or partial (±) agglutination through 1:200	Complete or partial agglutination through 1:400	Complete agglutination through 1:600	
			Absorbing strains							
154	12	8	19, 20, 21, 22, 24, 25		10	18				154-25
108	2	1					9			
100	2	1					3			
109	1	1					(1 and 2)			
157	2	1						3B		
152	2	1				6				
130	2	1		5						
J.S.	1	1							J. S.	
108	2	2	9, 14							108-9
154	1	1		25						
152	1	1			6					
140	4	2	1C	4						140-1 complex
170	1	1	3A		25					
154	13	3	18	10						154-18
154	3	3		10†, 18		25				154-10

* The lowest dilution was usually 1:50, but it was 1:25 in the case of sera with a titer 1:600 or less.

† Strain 10 never completely absorbed the agglutinins of its homologous serum.

other hand, we would not expect all agglutinins for strain 154-(10) to be absorbed by strain 154-(25), even without assuming the presence of specific agglutinins.

Reciprocal absorptions of the sera 154-(10) and 154-(18) are equally inconclusive. Strain 154-(10) absorbs from its homologous serum all the agglutinins for strain 154-(18) and strain 154-(18) does not absorb all agglutinins for 154-(10). However, any inferences one might draw from the above concerning the relationship of the two strains, are invalidated by the fact that strain 154-(10) had been partially agglutinated by normal serum from the same rabbit. The size of the serum dose used is also a factor to be considered as the dose was so large that the more sensitive strain 154-(10) was unable to absorb all the agglutinins from the homologous serum. The test with serum 154-(18) was carried out with a broth suspension of strain 154-(10) and happened to be fairly successful. In this case the agglutinins were removed for strain 154-(10) by absorption with that strain, but not entirely removed for strain 154-(18). Similarly, absorption by strain 154-(18) removed the agglutinins for that strain, but not for strain 154-(10). We seem justified in inferring that these strains, though closely related, are not quite identical.

In table 10 an attempt has here been made to classify the various strains tested according to the degree of agglutination produced with the homologous strains by the sera after absorption.

CONCLUSIONS

The tabulated results of our work as a whole justify us in coming to the following conclusions:

1. Direct cross-agglutination, even with strains of the same case, is not necessarily a proof of complete identity.

2. Cross-agglutinating strains from other cases absorb agglutinins in different amounts.

3. A striking metabolic characteristic, such as the production of green on blood, may be present in one strain and almost absent in another without necessarily indicating a wider divergence

than exists between strains agreeing in this characteristic, but chosen from different cases.

Our absorption tests with sera produced by several strains of the same case, lead us to conclude that we are not dealing with absolutely fixed type organisms. It is probable that the peculiar sensitivity of the antigen emphasizes small differences that might otherwise not be noticeable, but beyond this it seems indicated that alpha streptococci are organisms which easily undergo slight modifications. As might be expected, we find on the whole that dissimilarities are fewer and less marked in strains of the same than of different cases. We have enough closely related strains from different cases to suggest that some of these strains may have descended originally from the same parent organism, but these related strains are not sufficiently numerous, nor is their clinical distribution sufficiently exclusive for us to regard them as primary etiological agents in the influenza epidemic.

REFERENCES

(1) Williams, Unneberg, Goldman and Hussey: Jour. Immunol., 1921, **6**, 53.
(2) Smith and Brown: Jour. Med. Res., 1915, **31**, 455.
(3) Krumwiede and Valentine: Jour. Infect. Dis., 1916, **19**, 760.
(4) Tunnicliff, R.: Jour. Infect. Dis., 1920, **26**, 405.
(5) Rosenow, E. C.: Jour. Infect. Dis., 1920, **26**, 597.
(6) Valentine and Cooper: Jour. Immunol., 1919, **4**, 359.

ON THE PHOTOLABILITY OF SERUM COMPLEMENT[1]

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The purpose of this investigation was to discover the laws governing the destruction of complement (alexine) in normal serum by light. While the inactivation of the complement by other means, such as heat, shaking, and the chemical influences of acids and alkalis, have been already quantitatively studied and described, photo-inactivation has apparently been studied only qualitatively (8, 9, 10, 11, 12). It seemed to the writer, therefore, that the latter form of inactivation was worthy of a more detailed and exact investigation. While the work was in progress, the thorough researches of S. C. Brooks (1, 2, 3, 4, 5) on the same subject, though with somewhat different technic, came to my attention. Although we have covered much the same ground, I believe it may be of interest to compare the results obtained independently by different workers and by different methods.

TECHNIC

Complement. The complement used was exclusively undiluted normal swine-serum. The blood was caught in sterile glass cylinders and left for twenty-four hours at room temperature; the serum was then decanted and frozen at about −14°C. and left so until required for use. On those occasions when the serum has been employed at once, without being previously frozen, it is marked in the table as "fresh ser." Before use, the bottled frozen serum was melted in water at about 25°C.

[1] I beg here to express my sincere thanks for, and appreciation of the kind advice and all other assistance constantly given me by Dr. Th. Madsen, Director of the State Serum Institute in Copenhagen.

and immediately afterwards was put into an ice-bath until wanted for the experiments.

Radiation. For radiation there was employed a mercury-gas quartz lamp (from the "Quartzlampen Gesellschaft," Hanau), burning at 140-volt tension at the poles of the lamp and 4.3 amperes. It was estimated that the efficiency of the lamp would remain constant as long as the two current meters showed constant values; a state which was always reached within fifteen minutes after lighting and was usually maintained automatically, and seldom with the aid of a rheostate. The absolute light-strength was never measured, but a constant range of 150 mm. distance was maintained between the lamp and the serum, when not purposely varied in order to change the relative light-intensity.

The serum was exposed in a flat round dish, with a bottom of mirrorglass and a frame of the same material, covered with a plate of Jena uviolglass, 1 mm. thick, 12 cm. in free diameter and giving a clear space of 4.5 mm. between the two glasses. The box was held together by picin, a cement of asphalt and rubber, and in the circular frame there was an opening 7 cm. wide for the insertion of a glass rod, acting as the mixer of the fluid, and for the filling and cleaning of the interior and also to allow of successive samples being rapidly taken out by means of a pipette. The dish had a capacity of about 80 cc. but never held more than 60 cc. of serum. The whole was immersed to two-thirds of its depth in a water-bath with a motor-driven mixer, containing 30 litres, and was kept at a suitable constant temperature by means of a gas-flame or a current of cold water. The regulation was usually carried out by hand. The degree of accuracy has been noted in each experiment; the variations never exceeded ±0.4°C., remaining, as a rule, within 0.10°. The mixing-rod inside the serum-holder, 3 mm. thick and curved like a lancet-blade on a shaft, was moved to and fro by the motor at the rate of about 20 oscillations per minute. It left no part of the fluid at rest. As no foam was formed and the apparatus moved very gently, all destruction of complement by rocking may be regarded as excluded (Scaffidi, 12). The dish was fixed at an

angle of 45°, with the surface of the water somewhat higher than that of the serum. The lamp was placed with its tube horizontal, parallel to the edge of the free serum surface and as near to this as possible. The distance was measured from the nearest point of the tube to the center of gravity of the front serum surface. The degree of accuracy for this distance may be estimated at 0.3 cm.

Before radiating the serum in any experiment, the lamp-current and the bath-temperature were made constant. After this, serum was poured into the dish, which was then protected by means of an iron plate in front and a black paper behind, and the mixer started. A small quantity of serum (5 to 10 cc.) was put into a Jena-glass bottle in the same bath, light-sheltered, as a control of the degree of destruction by temperature ("heat control"). After exactly ten minutes—in less than which time, the serum was found, on testing, to have reached the temperature of the bath—the iron-plate was removed and the first sample, [O] was taken. After varying intervals successive samples were taken (each of about 2 cc.) and, when higher temperatures were reached, control samples were taken now and then from the bottle. These samples were immediately put into an ice-bath, where they remained, sheltered from light, until titration.

Titration. The titration was performed at once or some few hours afterwards. The method was a slight modification of that adopted by the Institute for hemolytic measurements (Madsen-Boas, 6), and was carried out principally as follows: There was employed for each sample a series of 10 to 12 tubes, calibrated with an exactitude of less than 5 per cent. In each tube there was put 0.5 cc. "sensitized" sheep-blood corpuscles, a variable quantity of the serum sample (complement) and 0.9 per cent NaCl solution, up to 1.25 cc. of total fluid. The blood-corpuscle fluid ("red cell suspension") was prepared from blood that had been centrifugated for ten minutes and washed with the same salt solution twice, after which 5 cc. of the centrifugate was diluted to 100 cc. This red cell suspension was sensitized with equal parts of a rabbit-sheep-hemolytic amboceptor dilution, just so strong that it contained 2.5 units. The

amboceptor titer had been tested beforehand with the aid of a guinea-pig complement, which gave quicker and more appreciable results than did swine-serum. Each tube was first filled with the salt solution, then with the serum (complement) or serum dilution; finally was added the sensitized red cell suspension. The tubes were immersed in an ice-bath during the pipetting of serum and blood, and afterwards were put into a water-thermostat at 37°C. for exactly two hours, after which they were placed in an ice-box (temperature 2–7°C.) until the following day, when the hemolysis in each tube was colorimetrically measured in daylight by means of a Madsen hemoglobin scale. A minor error occurred during the course of the earlier experiments in these color measurements, namely, that a layer of red solution often came next above the intact blood corpuscles, and a brighter layer topmost. On these occasions the hemolysis was read approximately. Comparisons made with a better technique showed that the mistake did not exceed 10 per cent. In the later experiments, the tubes were shaken before being placed in the colorimeter. This apparatus consisted of a black box with three vertical cells, separated by thin partitions. By three pairs of square holes the light passed through the fluid and tubes in the cells. But this shaking often clouded the tubes and made any reading impossible. Still later on, I had the tubes thoroughly shaken before their being placed in the ice-box for the night, with the result that the fluid was almost completely homogeneous in the morning. I attribute the better conformity of the results from the last period entirely to this technical detail.

The fluid-quantities used being one-fourth of the doses in the original method of Boas, the real amounts of serum (complement) in the glasses have always been multiplied by 4 when shown in the following tables. These last "whole dose" amounts make up the titer figures, p. They were always chosen in some suitable part of the following series of p = 3, 2, 1.4, 1, 0.70, 0.50, 0.30, 0.20, 0.14, 0.10, 0.07, 0.05, 0.03, 0.02, 0.01. The results are entered graphically on a coördinate system, the p's as abscissae, the hemolysis percentage, h, as the ordinate. These last have been corrected before being entered, viz., as regards

spontaneous hemolysis in a control O-glass, and also with respect to the serum's own color which, in the concentration $p = 1$, was found to be equal to 2–8 per cent hemolysis. I have also supposed that this color, which greatly resembles that of diluted hemoglobin adds to the value of the true hemolysis color. The error—if it be one—is probably not of any great importance.

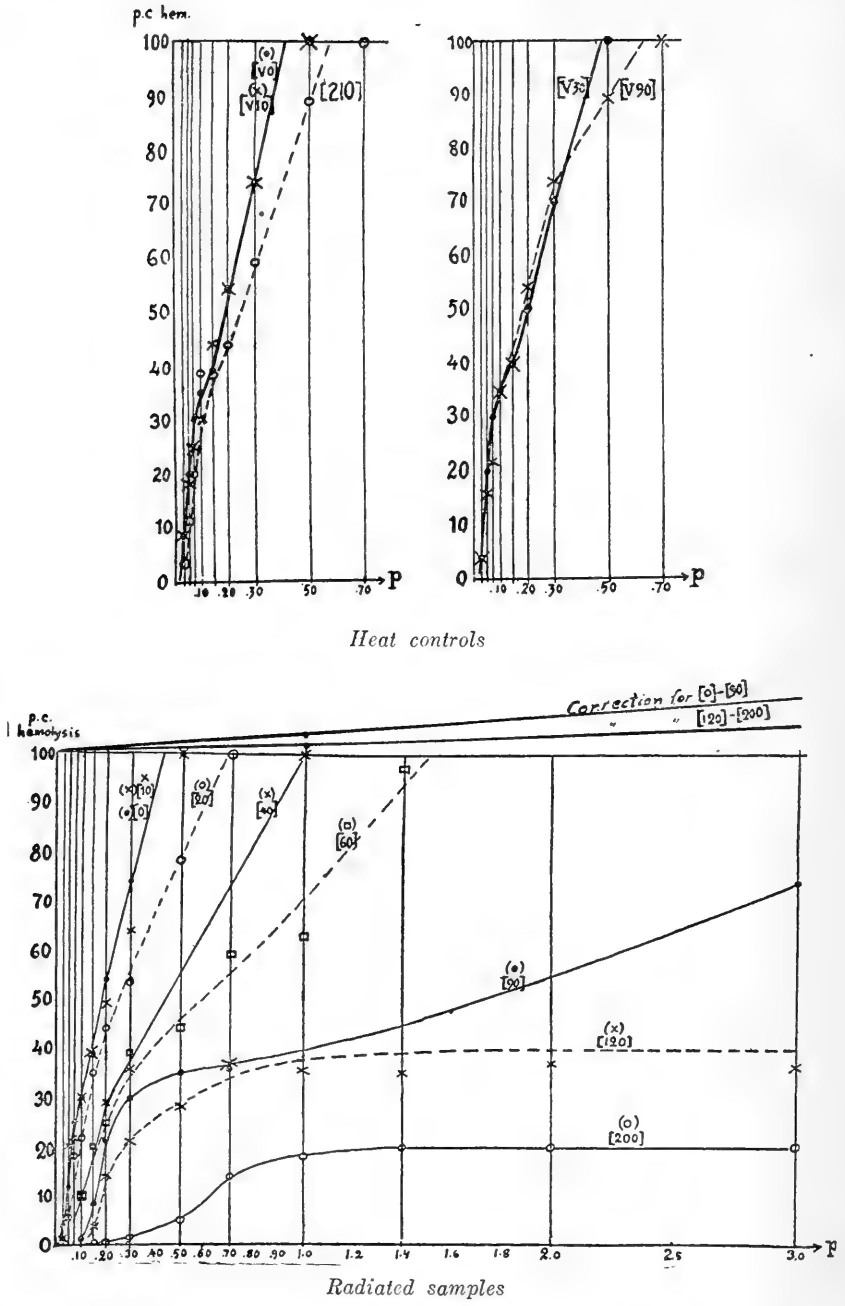
For the determination of the complementary strength by the aid of such a series of glasses, I have employed the following method:

With the help of the above-mentioned hemolysis curves (fig. 1) I look for the h-values, i.e., the corrected and most probable hemolysis percentages, for each of the employed serum concentrations, p, commencing at $p = 0.02$ and ending at $h = 100$ per cent. Each h is divided by its corresponding p. This fraction, h/p, indicates the efficiency-value of the serum mixture in question, i.e., the produced effect (h) per unit of agent (p). If, taking one curve, you sum up these fractions commencing, for instance, at $p = 0.02$, and divide each sum by the number of its terms, you obtain a mean value, $\frac{\Sigma h/p}{n}$; which is, however, not constant. It increases as you follow the calculation along the curve, and finally reaches a maximum value. I have employed this as a measure of the complement strength, S.

Being a function of the relation between effect and cause, and a greater number of points being used on the curve, this formula is more rational than the one I first employed, which was very similar to that of Brooks (4), which took the mean of the p-values for only five different hemolysis percentages. And, by comparison, it gives more equal values and smoother curves than the latter.

Furthermore, I have not adopted the relative values proposed by Brooks who takes a cold serum control as the unit strength for each separate experiment, because of the variations in strength this control exhibits during the time of experiment. It appears in general to increase in strength for some hours.

The S-values thus obtained are employed for drawing the destruction-curves (fig. 2), both for the radiated samples and also for the dark heat-controls, the S as ordinates, the time as abscissa.



Heat controls

Radiated samples

Fig. 1. Experiment XVI. Hemolysis Curves

The reaction-constant, k, is then calculated for each curve out of the observed values, employing the formula for monomolecular reactions:

$$k = \frac{\log \frac{a}{a - x}}{t \times \log e}$$

where, in this instance, a indicates the original complement force S_o; a-x the remaining force S_t, after the lapse of t minutes.

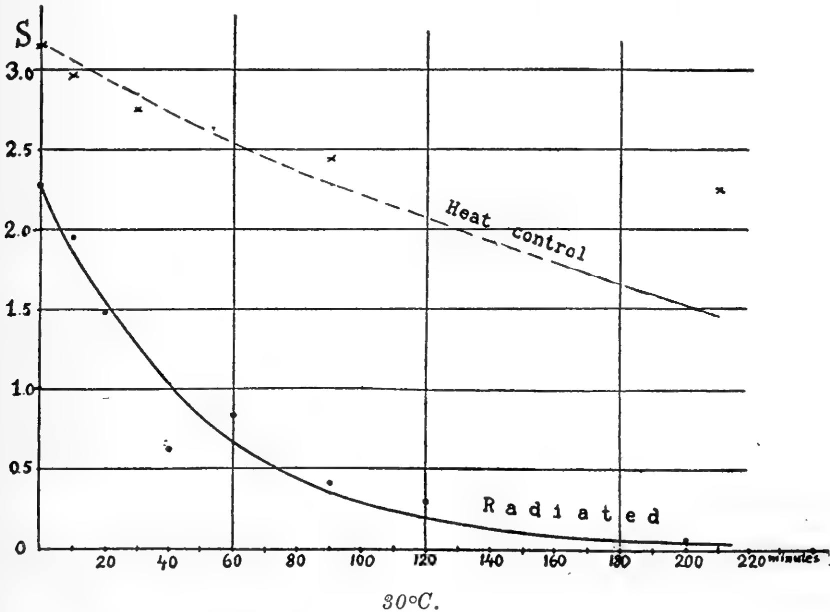


FIG. 2. EXPERIMENT XVI. REACTION TYPE AND SPEED

(e is the basis of the natural logarithms.) The symbol k thus gives a measure of observed destruction-speed, being the mean of the values for all the successive times. In order to discover the true photo-destruction speed, k_1, I have subtracted the destruction-speed of the thermo-control, k_v, from that of the radiated portion, k_r.

Wishing to find the influence of temperature on these reactions, three series were made on one day (experiments XIII, XV,

XVII) in order to find the k-values in the van't Hoff-Arrhenius formula:

$$\frac{k_1}{k_2} = e^{\frac{\mu}{2} \cdot \frac{T_1 - T_2}{T_1 T_2}}$$

where T_1, T_2 are the absolute temperatures in the two reactions running at the speed k_1 and k_2, and μ is the desired temperature-coefficient.

Together with these two investigations—the search for the reaction, law and speed, and for the temperature-influence—I have also made some experiments in order to see if the alteration of the lamp-distance really produces the effect one might expect beforehand: a change in k-magnitude inversely proportional to the square of the distance. Secondly, I have tried the influence of dilution. In all other experiments, I have radiated whole serum in spite of its great opacity to chemical rays which has caused such a slow destruction-speed, even in this thin and well-stirred layer, that the exposure necessary has been from forty-five minutes to two hours. Even if the dilution were made with balanced salt solution, it might essentially alter the complement, and could not, *a priori*, be regarded as a non-destructive operation.

A fairly large number of experiments were carried out, but only a few are reproduced here and of these only one is given in its entirety in order to show the method employed and the degree of error and uncertainty attending these extremely delicate investigations. The other experiments of the same kind have given the same or similar results, and the same method has been followed as that described here.

In the following tables, [0], [10], etc., mean samples taken from the dish 0, 10, etc., minutes after the commencement of exposure; [V10], [V60], etc., are samples from the heat-control bottle, taken 10, 60, etc., minutes after immersion in the bath. [*V*0] consequently, means the non-handled stock-serum kept at some few degrees above the freezing-point. The value of [*V*10] thus should be equal to that of [0].

The figures in the primary tables (experiments XVI, XIII: "hemolysis in per cent") show the observed hemolysis percentage without any correction, 100 or >100 means complete hemolysis (no cell sediment on the bottom); 100* means a color equal to 100 in the scale, with a cell sediment on the bottom. All figures below 100 indicate incomplete hemolysis. The values above 100 per cent are attributed to the color of serum itself.

EXPERIMENTS

Out of the several tests made to find out the reaction-type and speed, I have chosen one as a specimen, it being of considerable length, and showing a very regular course of the photo-inactivation. Here the primary data are given in full, so as to fully elucidate my procedure.

1. Reaction type and speed

Experiment XVI. Temperature 3.01° ±0. 15°. Distance: 15 cm. Fresh serum. Amboceptor titer: 0.20

From table 1 there have been drawn the hemolysis curves in figure 1, with corrections made for own-color of the serum, according to the correctionline, topmost in the figure. From each of these curves there has been calculated the complement-strength according to the formula, explained above:

$$S = \frac{\Sigma h/p}{n} \text{max.}$$

These values are reproduced in the following table 2 as $S_{obs.}$ and in the graphic representation of the reaction course in figure 2.

From them there is calculated the reaction-constant, k, in accordance with the monomolecular formula. The mean value is given for each of the radiated and the light-sheltered heat-control portions, k_r and k_v, respectively. $S_{calc.}$ shows the values calculated for a reaction with this mean value as reaction-constant, and the same initial value as the observed one.

TABLE 1

Hemolysis in per cent (direct readings)

p	RADIATED								HEAT CONTROLS				
	[0]	[10]	[20	[40]	[60]	[90]	[120]	[200]	[V0]	[V10]	[V30]	[V90]	[V210]
3.0						80	c40	c25					
2.0					>100	—	c45	c25					
1.4				>100	*100	—	c40	c22					
1.0				100	65	—	40	20					
0.70	>100	100	>100	50	60	37	40	14	>100	>100	>100	100	100
0.50	100	100	80	40	45	37	30	6	100	100	100	90	90
0.30	75	65	55	37	40	30	22	2	75	75	70	75	60
0.20	55	50	45	30	25	22	14	1	55	55	50	55	45
0.14	40	40	35	8	20	8	4	1	40	45	40	40	40
0.10	30	30	22	2	10	2	1	0	35	30	35	35	40
0.07	25	22	18	1	2	1	0		30	25	30	22	20
0.05	12	20	6	0	1				20	18	20	16	12
0.03	4	2	1	0					8	4	4	4	4
0.02	1	0	1						2	1	2	1	2
0.01	0	0							0	0	0	0	0

Controls with: (1) no serum, only sensitized blood = 0 per cent hemolysis (2) serum only, corresponding to p = 1.0, from sample

[V0]: 4
[20]: 4
[200]: 2
[V210]: 4

* Indicates incomplete hemolysis.

c Indicates approximate reading, because of opalescence and brownish tint.

TABLE 2

Complement strength

	IN RADIATED PORTION								IN HEAT CONTROL				
	After 0 minutes	After 10 minutes	After 20 minutes	After 40 minutes	After 60 minutes	After 90 minutes	After 120 minutes	After 200 minutes	After 0 minutes	After 10 minutes	After 30 minutes	After 90 minutes	After 210 minutes
S_{obs}	2.29	1.95	1.49	0.62	0.73	0.41	0.30	0.07	3.17	2.97	2.76	2.43	2.24
S_{calc}	2.29	1.86	1.52	1.02	0.67	0.36	0.20	0.04	3.17	3.05	2.84	2.28	1.46

K_r = 0.0203 mean; K_v = 0.0037 mean.

A comparison between the observed and the calculated values show that the light-destruction within the limits of experimental error can be expressed by the monomolecular formula. A number of other experiments of the same kind all show the same monomolecular type with the same degree of exactness.

Out of a number of experiment-series, where complement has been destroyed at various temperatures, I have chosen one (No. XIII) where the curves are specially smooth and easy to calculate. It should be observed that experiments with different temperatures, made on different days, are not comparable. The freezing of the stock serum during the night and other factors, may cause alterations which we cannot account for. Only those experiments, therefore, are used for determining the influence of temperature on the reaction speed which are carried out on the same day and with the same serum, and in quick succession, and under as similar conditions as possible. The following experiment shows a triple series, carried out at the temperatures: 46°, 36°, and 26°C.

2. Temperature influence

Experiment XIII.

Observed hemolysis

p	46° ± 0.3°							
	[0]	[10]	[20[	[35]	[50]	[*V*0]	[*V*10]	[*V*60]
2.0					65			95
1.4				90	60			95
1.0				65	55			95
0.70			100*	55	50	100	100	95
0.50	>100*	>100*	80	50	40	95	100	90
0.30	100*	70	55	40	35	95	95	90
0.20	65	60	40	35	22	70	95	90
0.14	50	45	35	22	16	40	65	65
0.10	35	30	22	16	6	40	40	35
0.07	30	22	18	4	2	30	30	30
0.05	18	14	10	1		22	18	16
0.03	8	6	3			10	12	10
0.02	4	4	1			5	10	8
0.01	1	0				1	1	1

Observed hemolysis—Continued

p	36.0° ± 0.10°						
	[0]	[10]	[24]	[40]	[60]	[V10]	[V70]
2.0					80		
1.4					65		
1.0				80	50		>100
0.70	>100	>100	100*	70	—	>100	>100
0.50	100	100	100*	55	45	>100	>100
0.30	100	90	60	45	35	>100*	100*
0.20	75	55	45	35	25	80	75
0.14	50	45	30	30	18	55	55
0.10	45	40	25	18	4	50	45
0.07	40	35	22	16	1	40	35
0.05	25	20	12	6	1	25	25
0.03	12	8	4	2		14	12
0.02	8	2	2	0		6	
0.01	0	0	0			0	

p	26.0° ± 0.15°							
	[0]	[10]	[20]	[40]	[80]	[V0]	[V10]	[V90]
2.0					80			
1.4					55			
1.0				100	50			100
0.70	100	100	100	85	45	100	100	100
0.50	100	100	100	65	40	100	100	100
0.30	100	70	70	50	30	100	100	100
0.20	45	55	55	40	22	55	70	70
0.14	55	50	45	25	16	50	55	60
0.10	30	18	40	22	8	25	40	50
0.07	30	30	25	14	2	35	30	30
0.05	—	16	12	6	1	22	20	25
0.03	—	4	—	4	0	14	14	20
0.02	—	0	1	1		12	10	8
0.01	—	0	0			1	1	0

Control with no complement:
0 hemolysis.
Control with serum only:
Sample [V0]: 1.
Sample [80]: 1.
* Indicates incomplete hemolysis.
>100 indicates deeper color than in scale tube "100."

From the titration curves, drawn in accordance with these figures, the following mean values are obtained and the curves of figure 3 drawn.

Titration results

		[0]	[10]	[20]	[40]	[80]	[V0]	[V10]	[V90]
26°	S_{obs}	5.0	2.19	2.95	1.46	0.59	5.0	5.0	5.0
	S_{calc}	5.0	3.78	2.86	1.63	0.53	5.0	5.0	5.0

K_r = 0.0280 mean; K_v = 0.

		[0]	[10]	[24]	[40]	[60]	[V10]	[V70]
36°	S_{obs}	3.93	2.83	1.90	1.17	0.57	4.13	3.87
	S_{calc}	3.93	2.87	1.85	1.13	0.60	4.13	3.87

K_r = 0.0311 mean; K_v = 0.0011.

		[0]	[10]	[20]	[35]	[50]	[V0]	[V10]	[V60]
46°	S_{obs}	3.0	2.33	1.82	0.80	0.53	4.30	4.24	4.00
	S_{calc}	3.0	2.22	1.63	1.04	0.66	4.30	4.25	4.05

K_r = 0.0305 mean; K_v = 0.0010.

"$S_{calc.}$" shows the complement-strength values calculated under the presupposition that the reaction follows the monomolecular formula and that it has the reaction-constant k_r, obtained as the mean of the observed complement-values S_{obs}.[2] k_v gives the reaction-constant for the thermo-destruction at each temperature. If this constant is subtracted from the corresponding light destruction-constant k_r, we obtain the following values for the pure photo-reaction speed, k_l: at 26° 0.0280; at 36°, 0.0300; at 46°, 0.0295. As these values show a very irregular course in relation to the temperature, no certain temperature coefficient can be calculated from them. If there is any numerical value of such a coefficient, it must be low, or even negative.

The same result is gained from two other experiments, reproduced here, although only as regards their final figures:

[2] In the experiment at 26°, the 10-value, being evidently due to one single mistake in titration, has been altogether neglected in the calculation.

	EXPERIMENT XV				EXPERIMENT XVII		
	k_r	k_v	k_l		k_r	k_v	k_l
°C.				°C.			
15	0.0242	0.0	0.0242	15	0.0233	0.0	0.0233
30	0.0193	0.0	0.0193	30	0.0190	0.0006	0.0184
45	0.0420	0.0060	0.0360	45	0.0330	0.0010	0.0320

Even here, the differences being small and irregular, it may be concluded as a whole that the temperature-influence on these reactions is very small or negative.

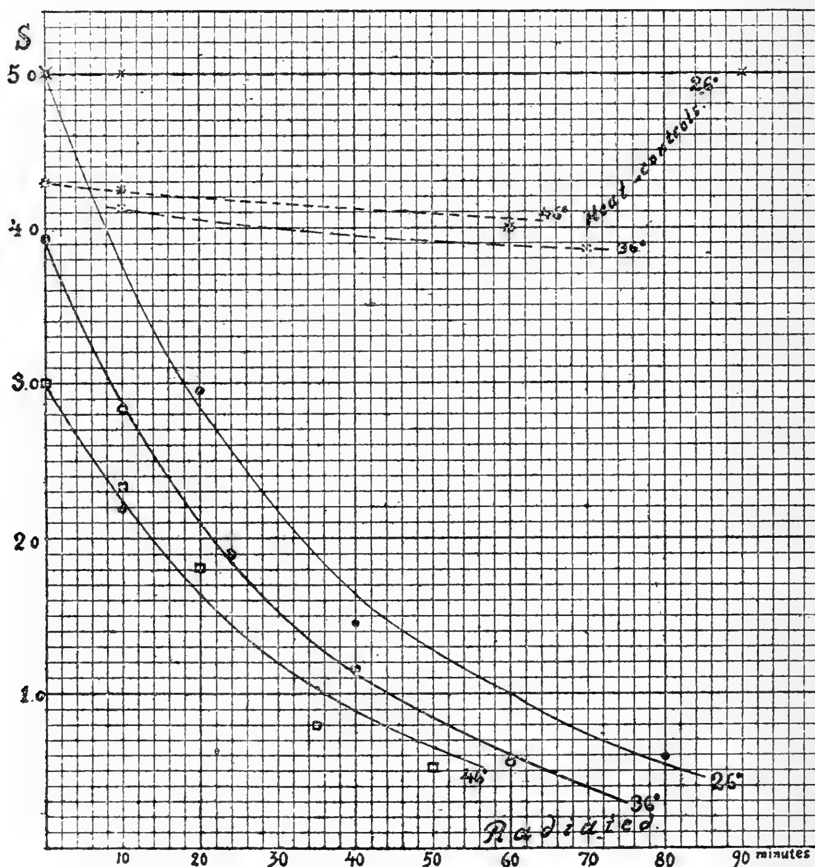


FIG. 3. TEMPERATURE INFLUENCE

The following experiment is for the purpose of discovering whether a change in light-intensity alters the reaction-speed in the same proportion. For this purpose I have chosen to change the radiation-range in the proportion 1:2, which signifies an alteration in the light-intensity from 1 to $\frac{1}{2}$. The temperature was, all the time, constant, and the same serum was employed.

3. *Influence of light-intensity*

Experiment XVIII. 45° ± 0.2°. Fresh serum.

1. Distance = 15 cm. (Light-intensity = 1)

	RADIATED					
Minutes	0	10	20	30	45	60
S_{obs}	2.27	1.47	1.06	0.74	0.51	0.36
S_{calc}	2.27	1.62	1.16	0.81	0.50	0.30

K_{e} = 0.0336 mean.

2. Distance = 21.2 cm. (Light-intensity = $\frac{1}{2}$)

	RADIATED							
Minutes	0	15	30	45	60	80	100	120
S_{obs}	1.57	1.31	1.01	0.80	0.60	0.39	0.22	0.15
S_{calc}	1.57	1.23	0.97	0.76	0.66	0.43	0.31	0.23

$K_{\frac{1}{2}}°$ = 0.0162 mean.

3. Heat-control

Minutes	0	130	245
S_{obs}	2.03	1.18	0.63
S_{calc}	2.03	1.14	0.68

K_{v} = 0.0045.

The observed values agree fairly well with those calculated according to the monomolecular formula.

If the thermo-inactivation speed-constant k_{v}, is subtracted from the photo-inactivation speed-constants k_{1} and $k_{\frac{1}{2}}$, the true light-destruction speeds are obtained, viz.: at light-intensity $1:k_{1}$ 0.0291; at light-intensity $\frac{1}{2}:k_{\frac{1}{2}}$ 0.0117.

The proportion $\frac{k_1}{k_{\frac{1}{2}}}$ is equal to 2.49 instead of the expected 2. The difference may possibly be regarded as lying within the experimental error.

Finally, I wished to determine whether it was feasible to dilute serum before radiating it, and to reach the same results as with whole-serum, only at a higher speed, in proportion to the amount of dilution. From the phenomenon of hydro-inactivation of complement, I divined that even dilution with a balanced salt solution might alter the nature of complement, thus causing not only a proportionally lower strength of the fluid, and a proportionally more rapid destruction, in consequence of the greater transparency of the dilution, but also some unexpected change. For this purpose, whole-serum, a 1:3 dilution, and a 1:10-dilution, were radiated on the same day and under entirely similar conditions.

4. Influence of dilution

Experiment XIX. 39.95° ± 0.20°. Fresh serum.

A. Undiluted serum

	[0]	[10]	[25]	[40]	[60]	[V0]	[V70]
S_{obs}	7.91	4.65	2.88	1.76	1.20	7.91	4.65
S_{calc}	7.91	5.10	2.61	1.35	0.56		

$K_{\frac{1}{4}}$ = 0.0442 mean; K = 0.0078.

B. Serum diluted 1:3

	[0]	[5]	[10[	[15]	[25]	[V0]	[V35]
S_{obs}	0.88	0.65	0.53	0.44	0.26	1.08	0.83
S_{calc}	0.88	0.68	0.53	0.40	0.24		

K_r = 0.0518 mean; K_v = 0.0071.

C. Serum diluted 1:10

	[0]	[2]	[4]	[6]	[8]	[12]	[V0]	[V22]
S_{obs}	0.22	0.17	0.16	0.13	0.11	0.08	0.26	0.19
S_{calc}	0.22	0.18	0.15	0.12	0.10	0.07		

K_r = 0.0975; K_v = 0.0143.

Note: In (B) the directly found S-values are multiplied by the dilution-cipher 3 before being entered in this table and on figure 4. In (C) they are multiplied by 10. The destruction-curves thus show the real strength of complement. In spite of this operation, it is found that the diluted sera are disproportionally weaker than the whole-serum. But all of them follow satisfactorily the monomolecular formula, as found by the calculated values.

The proportion between the reaction-speeds, after subtracting the thermo-constants, is found to be:

	k_1	REACTION-SPEED EXPRESSED IN PER CENT
Undiluted serum	0.0364	100
Serum diluted 1:3	0.0447	123
Serum diluted 1:10	0.0832	228

There evidently exists no parallelism between dilution and reaction-speed, although the latter increases with the former.

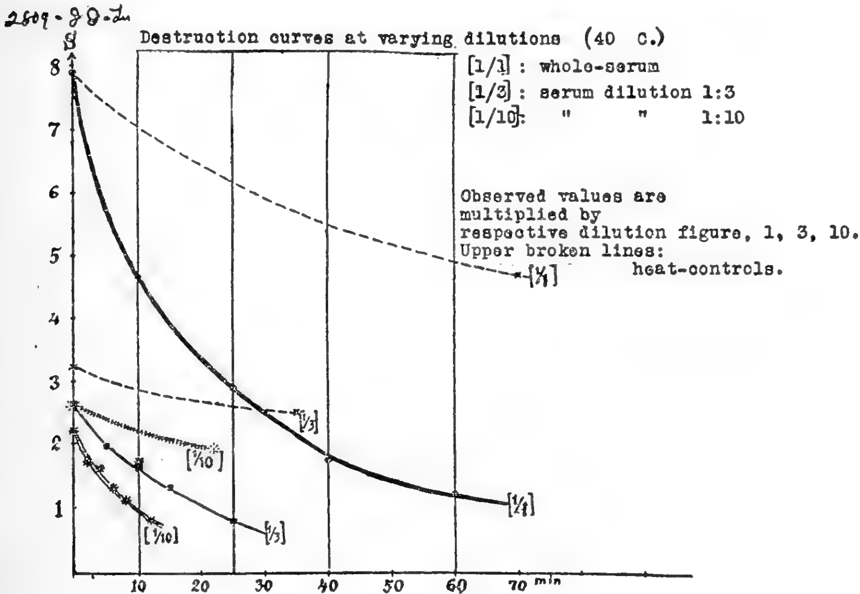


FIG. 4. EXPERIMENT XIX

SUMMARY

There has been shown by these experiments that:

1. The light-destruction may be fairly well expressed by the monomolecular formula.

2. The rate of the destruction is low, k varying from 0.019 to 0.050.

3. The temperature-influence is small or none. μ certainly does not exceed two or three thousandths—which signifies an increase in k of 1.1 to 1.7 per cent for 1°C. increase of temperature at 27°C.

4. Variation in lamp-distance causes changes in reaction-speed, which do not differ essentially from inverse proportionality to the light-intensity.

5. Dilution of serum before radiating causes not only the expected decrease of complement strength, but also alters very irregularly its magnitude. It causes, too, an increase in reaction-speed, which, however, is not parallel to the dilution-amount.

6. The color of serum changes obviously during radiation, from orange to dull yellow, the opalescence increasing at the same time. This is accounted for in the colorimetry.

7. As regards the exactness of the measurements made with this method, the primary figures have a possible error of about $\pm$ 10 per cent. The graphically found values may be regarded as more exact (about 5 per cent possible error). The k- values are chiefly found to differ within $\pm$ 12 per cent from the mean, which may be regarded as quite satisfactory. The temperature-variation in each experiment—being within $\pm$ 0.4°—may be disregarded in consequence of the insignificant temperature-influence.

REFERENCES

(1) Brooks, S. C.: Jour. Med. Research, 1918, **38**, 345.
(2) Brooks, S. C.: Jour. Med. Research, 1918, **38**, 345.
(3) Brooks, S. C.: Jour. Med. Research, 1920, **41**, 411.
(4) Brooks, S. C.: Jour. Med. Research, 1920, **41**, 399.
(5) Brooks, S. C.: Jour. Gen. Physiology, 1920, **3**, 169.
(6) Boas, H.: Wassermann Reaktion, Ref. in Communications de l'Inst. Serotherap. de l'Etat Danois, 1909 and 1911.

(7) Madsen and Watabiki: Oversigt af Kong. Danske Vidensk. Selsk. Forhandl., 1915, p. 125.
(8) Baroni, V., and Jonesco-Mihaiesti, C.: Compt. Rend. Soc. Biol., 1910, **68**, 393; **69**, 273.
(9) Courmont, P., Nogier and Dufour, A., Compt. Rend. Soc. Biol., 1913, **74**, 1152.
(10) Doerr, R., and Moldovan, J.: Wiener klin. Wochenschr., 1911, **24**, 55.
(11) Abelin, S., and Stiner, O.: Zeitschr. f. Immunitätforschung, 1913, Orig., **19**, 1.
(12) Schaffidi, V.: Biochem. Zeitschr., 1915, **69**, 162.

PROPHYLACTIC TREATMENT FOR RABIES BY MEANS OF STANDARDIZED GLYCERINATED VIRUS

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In a previous article (1) we compared the various antirabic methods, discussed their gradual evolution from the crude procedure employed by Pasteur to a much more accurate and, judging from our experience, more efficient technic—that is, the use of a "standardized" virus in such a form that it can be preserved with practically unchanged virulence, for a long period of time.

The Harris (2) method of desiccating and preserving fixed virus is almost ideal from the standpoint of therapeutic efficiency, but it is cumbersome and complicated by many manipulations which afford opportunitites for accidental contamination of the virus during its preparation. The number of living infectious units in the finished product is also greatly affected by any variation in the rapidity of drying; as a result, after standardization and sterility tests have been made, much of the material prepared with so great effort, must be discarded. Some persons working with this method have had difficulty in maintaining the virulence of the virus. After many generations of storage the incubation period seems to have a tendency to lengthen.

We determined, therefore, to try to find an equally efficient method, free from these objections, of preserving the virus in a living state.

An analysis of the many papers on the subject suggests that heat, oxygen, light, moisture and various chemicals are the agents which cause rabies vaccines as they are usually prepared, to lose their virulence; that when these factors are not present the virus remains active for amazingly long periods of time.

Pasteur himself (3) sealed rabies virus in glass tubes by means of a blow pipe and noted that it maintained its virulence for several weeks in the summer heat. Because the oxygen in the tube was very soon reduced by the fresh tissue an anaerobic condition resulted. This was the first demonstration of the anaerobic preservation of rabies virus.

To Roux (4) belongs the credit of the discovery that fixed virus retains its virulence when placed in glycerine: Calmette (5) put the discovery to practical use for preserving the cords used in the classical Pasteur method, so that their usefulness was extended. This method is still in use in a large number of laboratories in this country and on the continent of Europe.

We believe that the preservative action of glycerin depends chiefly on the fact that oxygen is only slightly soluble in it, as shown by the following figures compiled from Müller (6).

PER CENT OF GLYCERIN	COEFFICIENT OF ABSORPTION	SOLUBILITY (VOLUME)	SOLUBILITY (WEIGHT)
20.5	0.02742	0.02890	0.000043
25.0	0.02521	0.02659	0.000040
37.3	0.02022	0.02133	0.000032
45.0	0.01744	0.01840	0.000027
52.0	0.01570	0.01656	0.000025
71.5	0.00950	0.01002	0.000015
88.5	0.00886	0.00935	0.000014

Temperature, 15.0°C.; pressure, 760 mm.

Glycerin rapidly extracts moisture, so that the moisture content of fixed virus emulsified in glycerin, is so reduced that its destructive action on the vitality of the virus is overcome. A suspension of fixed virus in glycerin, which contains a very even distribution of infective units, is readily obtained, probably on account of the peculiar diffusibility of the virus in glycerin as shown by Remlinger (7). On account of its antiseptic properties, glycerin gradually destroys many contaminating organisms, but in practice it is best to ignore this because tests have convinced us that this quality is greatly overrated and that many organisms retain their vitality for months when stored in glycerin.

For these reasons we decided to determine for ourselves the keeping qualities of rabies virus suspended in glycerin, despite the rather unfavorable observations of others summarized by Stimson (8).

Our present technic was evolved from a mass of experimental work, but we shall not burden this paper with unessential tables and details. This technic has now been in use for five years, and after forty-six passages our virus still retains its original virulence. For prophylactic treatments we do not use virus which has been in storage over six months. At times we have noticed in rabbits inoculated with our stored virus, a slight tendency toward lengthening of the incubation period, but as we have not used such virus for subsequent passages we have had no difficulty in maintaining a virus of fixed virulence.

Young rabbits from a known source are inoculated intracerebrally with from 0.015 to 0.075 mgm. of fixed virus suspended in salt solution. When an animal is completely paralyzed and appears to be moribund it is killed by bleeding. This reduces the amount of blood in the brain and lessens the amount of foreign protein in each therapeutic dose, so that it produces less local reaction than we observed when using either the old cord method or the brains from rabbits which had been killed by drowning. To reduce the protein further we use the brain alone because the cord contains fewer infection units in proportion to its weight.

After its removal, the brain is weighed and rubbed to a smooth paste in a mortar; then glycerin is slowly added, care being taken to incorporate each addition thoroughly before more is added. This process is continued until the total volume of fixed virus and glycerin has reached such a point that each 0.1 cc. of the suspension contains 15 mgm. of the fresh fixed virus.

Various sized amber glass ampoules are then filled up well into their necks with the glycerinated virus. Each ampoule is put into a separate test tube which contains a small pledget of non-absorbent cotton to protect the neck of the ampoule from breakage; a plug of this cotton and below it a wad of absorbent cotton are inserted into the tube to hold the ampoule in place. The tubes are placed in cold storage for a few hours. The mouth of

the test tube is then crowded full of pyrogallic acid by ramming the tube into this material. The test tube is then inverted and 2 to 3 cc. of 40 per cent caustic potash solution are added, followed by a pledget of absorbent cotton, and a good quality of rubber stopper is tightly inserted at once. If the ampoules are properly filled and the contents chilled the glycerinated virus will not change its position or escape from the ampoule. The object of

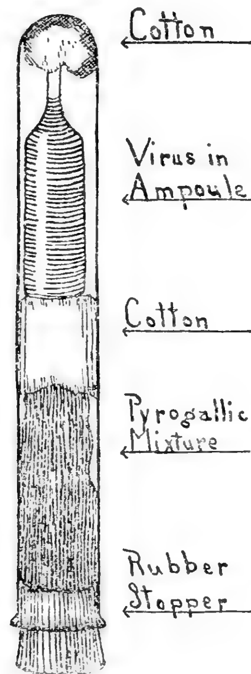

Fig. 1. Storage Unit

this procedure is to absorb the oxygen in the tube. A label with the serial number of the rabbit, the date and the exact amount of glycerinated virus contained is placed on each tube. These units are stored upright in test tube baskets at $-2°$ to $-4°$C. until required. The arrangement of each storage unit is shown in figure 1.

In addition to storage in the dark, the use of amber ampoules is a protection against the action of light.

Dilutions of the glycerin in distilled water are tested for the hydrogen ion concentration by means of the potentiometer method in order that we may be certain of its neutrality. Since glycerin is hygroscopic it must be kept tightly stoppered, and cannot be sterilized in the autoclave. We have found 180 dry heat for two hours to be satisfactory. Contact with many metals, specially aluminum that has been sterilized in the autoclave, exerts a deleterious action on the virus, therefore glycerin should be stored in glass containers, not in cans, and the ampoules should be filled with glass syringes to which gold canulas are attached.

The ampoules should be tested to see that the glass contains no soluble alkali by grinding samples in a mortar, adding distilled water, grinding further and then using phenolphthalein as an indicator.

We have found no antiseptic suitable for use in these emulsions, even 0.25 to 0.5 per cent phenol destroys their infectivity with comparative rapidity.

Throughout the procedure the most scrupulous precautions are taken to insure sterility and the usual tests of the fresh brain and of the emulsions are always made. The sterility tests used are those required by the U. S. Hygienic Laboratory for licensed manufacturers.

Table 1 shows the keeping qualities of the virus stored in this way. The apparent increase in infectivity after storage is rather constant and is probably due to a more uniform diffusion of the virus and not to its multiplication, although Pirone (10) interprets the infectivity of glycerin in which the brain of a rabid animal has been stored, as being due to the "cultivation of the virus."

The object of filling the ampoules well into their necks is to present as small a surface as possible for the solution of any oxygen which might remain in the tubes or subsequently get into them. In fact, ampoules filled in this way and kept in our cold storage for eight months, show no change in virulence even when the oxygen has not been absorbed from the test tube. Agitation of the tube does not cause the emulsion to dissolve oxygen for the glycerin solution is too heavy to alter its position readily when the ampoules are thus filled. If the glycerin has been sterilized

TABLE 1

DATE OF TEST	LABORATORY RECORD NUMBER	AGE OF MATERIAL	NUMBER OF RABBITS	DOSAGE	RESULTS
1917		*days*		*mgm.*	
June 13	212	0	6	A 1.5 B 0.3 C 0.1 D 0.015 E 0.003 F 0.0015	A died seventh day B paralyzed seventh day C paralyzed seventh day D paralyzed eighth day E paralyzed ninth day F survived
June 15	213	2	6	A 0.15 B 0.03 C 0.01 D 0.075 E 0.015 F 0.0015	A paralyzed sixth day B paralyzed sixth day C paralyzed seventh day D paralyzed seventh day E paralyzed eighth day F survived
July 10	217	27	7	A 3.0 B 1.5 C 0.3 D 0.03 E 0.015 F 0.005 G 0.0015	A paralyzed sixth day B paralyzed sixth day C paralyzed seventh day D paralyzed seventh day E paralyzed eighth day F paralyzed ninth day G survived
August 13	223	61	3	A 0.15 B 0.015 C 0.0015	A paralyzed seventh day B paralyzed seventh day C died ninth day
September 9	225	87	3	A 0.15 B 0.015 C 0.0015	A paralyzed seventh day B paralyzed eighth day C died eighth day
October 12	231	120	3	A 0.15 B 0.015 C 0.0015	A paralyzed sixth day B paralyzed seventh day C died eighth day
1918					
January 6	329	206	2	A 0.015 B 0.0015	A paralyzed sixth day B paralyzed sixth day
March 7	243	266	3	A 0.15 B 0.015 C 0.0015	A paralyzed seventh day B paralyzed seventh day C survived
June 5	250	356	2	A 0.015 B 0.0015	A died sixth day B paralyzed seventh day
1919					
July 24	276	770	2	A 0.015 B 0.0075	A paralyzed seventh day B paralyzed eighth day

in the autoclave, or if the ampoules are only partially filled and the tubes are occasionally shaken while in storage so as to present new surfaces of the emulsion to the oxygen, a marked decrease in infectivity commences in about the sixth month. When the glycerinated emulsion in control tubes is tinged with methylene blue, the appearance of a permanent blue color in the suspension corresponds closely to the decline in infectivity of the virus used for the tests. The loss in infectivity in partially filled ampoules, as well as in those in which the glycerin contains a considerable percentage of water due to stream, sterilization, can be prevented by absorption of the oxygen by means of the pyrogallic acid mixture. Taking all these things into consideration we still use caustic potash and pyrogallic acid in all of our storage tubes as an added precaution, even though this is probably unnecessary.

To make the dilutions used in the treatment of cases, a storage tube is selected which contains sufficient virus for the required number of doses. A nick is made in the test tube with a file, and the tube is broken so as to remove the ampoule without contamination. The contents of the ampoule are flushed out into the proper amount of 0.85 per cent salt solution, to which 0.5 per cent phenol has been added and the resulting mixture is trained through sterile gauze to remove shreds of the meninges.

The individual treatments may be sent in ampoules, syringes or vials to places near the laboratory. For shipments to a distance any of the methods now employed by the commercial houses, in which the glycerinated virus and salt solution are sent in separate containers to be mixed immediately before being injected into the patient, may be adopted.

Our strain of fixed virus was obtained in 1910 from the Research Laboratory of New York City Health Department. At present its average minimal lethal dose is about 0.015 mgm. This almost invariably kills the rabbits on time; a dose of 0.0075 mgm. may kill on time or it may show a delay of twelve to twenty-four hours.

It should be borne in mind that we are not working with toxins; with the same lot of virus the so-called minimal lethal or infec-

tious doses show greater variation than do toxins. Beyond a certain point of dilution the incubation period is lengthened with a fair degree of constancy. We have often killed rabbits by an injection of 0.0015 mgm.; with this high dilution the incubation period may be lengthened to even nine or more days. We regard the standarizing dose as the minimal concentration of virus which will kill a rabbit in as short, or nearly as short a time as heavy concentrations and we use this as a unit for standardizing dosage in the treatment of patients. A rabbit should be inoculated with 0.015 mgm. of each lot of virus for the purpose of standardization, and any lot of virus should be discarded if this dose does not cause the rabbit to become moribund by the end of the eighth day. Any test rabbits which are completely paralyzed by this time can be used for manufacturing the virus for treatment.

Rabbits should never be killed until they are apparently moribund if one wishes to secure a virus of the greatest concentration.

We were greatly impressed with the reasons advanced by Dr. Harris (11) in determining his treatment dosage, and we decided to follow him very closely in this. Since 0.1 cc. of our glycerinated virus contains 15 mgm. equivalent to approximately 1000 units (so-called minimal lethal or infectious doses) this amount is diluted with enough salt solution, containing 0.5 per cent phenol, to bring the quantity up to 2 cc. This is the average daily dose from the fourth day after instituting treatment, and is continued from eleven to nineteen days according to the severity of the bites and their location. In children under five years of age the average daily dose is 1.5 cc. In some very severe face injuries, a larger dosage, as high as 3.5 cc. to 4 cc. daily, is given during the first week. These dilutions are made daily, as rabies virus in phenolized salt solution is viable for only a limited time. On account of the impossibility of standardizing the virus with absolute accuracy, we insure an approximate average in the total dosage of each case by using virus from a number of different rabbits in making up the successive days treatment.

For the first three days 60 mgm. of dead fixed virus is given daily in all cases. This is prepared by making a double strength mixture of the glycerinated virus in salt solution containing

0.5 per cent phenol; the dose of which is 4 cc. on each of the first three days. The filled ampoules are placed in the incubator at 37°C. for twenty-four hours. During this time they should be agitated several times, otherwise the reducing power of the brain tissue, which is not destroyed by its sojourn in glycerin, will keep the bottom of the containers in an anaerobic condition and some of the virus may survive. The use of virus rendered avirulent by carbolic acid was first adopted in 1906 by Fermi (12), but we have modified his technic. The reason for commencing treatment with some form of dead virus has been aptly stated by Harris. The practical usefulness of dead virus as an immunizing agent has been shown by Cumming (13) and Semple (14). We can find no difference in experimental results between the Cumming "dialyzed virus" and carbolized material.

The Hygienic Laboratory places a seven-day expiration period on all living virus which does not contain more than 50 per cent of glycerin when emulsified for use. The keeping qualities of these emulsions will be made the subject of a separate paper; however, we wish to state briefly that when the emulsions have been shipped to a distant point in the summer, many persons who were supposed to have been treated with living virus have really received injections of virus which has lost its infectivity. Since, taken all in all, the results from these treatments have been excellent, this constitutes another argument to support the immunizing value of dead virus.

As Stimson states, "Results are better as the older formulae of treatment are replaced by those in which more virulent material is administered." We agree with the majority of authorities that in all cases of significant injuries the dead virus should be followed by an adequate course of injections of living virus, Of course some authorities object to the use of living virus on account of the danger of treatment paralysis following its use. The academic nature of this objection when virus containing a minimum amount of nerve material is used has been shown in a previous paper (15).

The clinical results following this method of preventive treatment are shown in these tables.

The treatment in most of these cases was sent to the family physician for administration.

As is to be expected these results are very similar to those recently reported by D'Aunoy (16), who used much the same dosage prepared by the Harris method. The mortality is far less than when we were using the classical Pasteur method, as modified by the Hygienic Laboratory, eight deaths in 1680 cases.

TABLE 2

Survey of cases

Total cases treated	1540
Number of deaths during treatment	0
Number of deaths within 14 days after completion of treatment	1
Number of deaths more than 14 days after treatment	0

TABLE 3

Location of injuries

Head and neck	155
Multiple bites of head and other parts	16
Multiple bites on several parts, not on head	45
Hands or arms	848
Leg or foot	279
Trunk	25
No data obtainable	172

TABLE 4

Diagnosis of biting animal

Laboratory diagnosis showing Negri bodies	626
Clinical symptoms of rabies	584
Not made (animal escaped)	158

In addition to these human cases, we have given like treatments to twenty-nine dogs. One of these died of rabies on the fifteenth day after commencing treatment, or the twenty-first day after having been bitten on the head. None of the others contracted rabies. On account of the disastrous results which may follow a failure of the Pasteur treatment in a dog, we have always discouraged it. We consider the use of shorter time treatments especially reprehensible, as the almost invariable result is that

the dog is freed from quarantine and thought to be safe before the real danger begins.

Our experience in immunizing other animals with short treatments in the period between November 12, 1912, and January 1, 1922, is shown in the following table. We gave these animals just ten times the usual daily human dose, for ten days. One case of treatment paralysis developed in a horse which promptly recovered.

As two cows treated in this way developed rabies it would seem even with this intensive treatment that ten days is rather less time than should be used to secure the greatest possible efficiency in the treatment of animals. However, this is probably the longest duration of treatment in animals which is possible from an

TABLE 5

KIND OF ANIMAL	NUMBER	RESULT
Horses	39	Protected to date
Mules	1	Protected to date
Hogs	26	Protected to date
Cattle	101	2 died of rabies, others protected

economic standpoint. When dealing with human life there is no economic standpoint.

We believe that our technic has solved the question of an economical and permanent standarized supply of a potent virus. The remaining problems are to be found in the dosage and the duration of the treatment. As the dosage which we have given has been so efficacious and has not proved dangerous, we have hesitated to make a change. Certain clinical observations of the results of treatment in men and animals have deterred us from shortening the treatment. The one fatal case (table 2) was a severe laceration above the eye, so large as to require several stitches to close it. The physician to whom we sent the treatment neglected to answer any inquiries and we did not receive any information until symptoms had appeared. This boy, of ten years, had been bitten four days before treatment was commenced. The wound had been treated only with nitrate of silver. The inoculations

lasted eighteen days, 15 mgm. of living virus being the daily dose after the usual first three days treatment with dead virus. Symptoms commenced one week after completion of treatment and the boy died three days later. Examination of his brain showed Negri bodies. We believe that a more prolonged and more intensive form of treatment might have saved this child in spite of inadequate cauterization and the fact that the wound was sutured.

TABLE 6

ANIMAL	LOCATION OF BITE	DATE OF BITE	TREATMENT STARTED	RESULT
Cow 1	Nose	November 22	November 24	Died December 15
Cow 2	Nose	November 22	November 24	Died December 17
Cow 3	Nose	November 22	November 24	Died December 17
Cow 4	Nose	November 22	November 24	Died December 19
Cow 5	Nose	November 22	November 24	Died December 19
Cow 6	Nose	November 22	November 24	Died December 19

Animals all bitten by the same dog, and were in their stations at the time. Treated by Drs. Failor and Morris, Veterinarians, Lima, Ohio.

TABLE 7

ANIMAL	LOCATION OF BITE	DATE OF BITE	TREATMENT STARTED	RESULT
Horse	Nose	March 4	March 6	Died March 25
Dog	Hind leg	March 4	March 7	Died March 29
Dog	Hip	March 5	March 8	Died April 12
Dog	Fore leg	March 5	March 9	Died April 7
Dog	No visible marks	March 5	March 10	Died April 20

Two hogs and three dogs bitten by the same dog but not treated also died of rabies. Animals treated by Dr. Wise, Veterinarian, Medina, Ohio.

When thinking of shortening the treatment in man, the occasional failures which occur in animals which have been given a six-day intensive treatment should be considered carefully. As an example of these we are giving two tables, in each of which animals were treated with virus furnished by different commercial houses respectively.

Many attempts to measure the degree of protection which can be conferred on rabbits by the use of varying numbers of

"minimal infectious doses" inoculated intracerebrally, have convinced us that these results are too variable to be dependable. The same can be said of the titration of the serum of protected animals for rabicidal properties. Therefore the clinical use of a method in a large series of cases over a long period of time, is the only reliable test, and changes in the length and intensity of treatment must be undertaken cautiously.

REFERENCES

(1) Phillips, J. M.: Ky. Med. Jour., 1921, **19**, 278.
(2) Harris, D. L.: Jour. Amer. Med. Assoc., 1913, **67**, 923.
(3) Pasteur's System, Hydrophobia, Chatto and Windus, 1887, 46.
(4) Roux, E.: Annales de l'Institut Pasteur, 1887, **1**, 87.
(5) Calmette, A.: Annales de l'Institut Pasteur, 1891, **5**, 633.
(6) Müller: Zeitschr. für Physikalische Chemie, **81**, 483.
(7) Remlinger, P.: Annales de l'Institut Pasteur, 1919, **33**, 28. Comptes Rendus Soc. de Biol. 1918, **81**, 20.
(8) Stimson, A. M.: Bull. No. 65, Hyg. Lab. U. S. Pub. Health and Mar. Hosp. Serv., Wash., p. 57.
(9) Anderson, R. P.: Jour. Ind. Eng. Chem. 1915, **7**, 587.
(10) Pirone, R.: La Riforma Medica, 1920, **36**, 782.
(11) Harris, D. L.: Jour. Inf. Dis. 1913, **13**, 155.
(12) Fermi: Il nuovo metodo Italiano per la cura antirabbica. Rome, 1916. Ann. d'igiene, **26**, Suppl.
(13) Cumming, J. G.: Jour. Inf. Dis. 1914, **14**, 33.
(14) Semple, D.: Scientific Memoirs, Officers of Med. and San. Depts. of Gov. of India, 1911, **44**, 32.
(15) Phillips, J. M.: Berry, F. and Snook, J. H. Jour. Inf. Dis., 1921, **29**, 97.
(16) D'Aunoy, R.: Jour. Inf. Dis., 1921, **29**, 261.

A STUDY OF THE PRECIPITIN AND COMPLEMENT FIXATION REACTIONS WITH TUBERCULOUS EXUDATES WITH SPECIAL REFERENCE TO TUBERCULOUS PLEURITIS

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This investigation was undertaken for the purpose of determining whether the immunological reactions of precipitation and complement fixation occur with pleural exudates of tuberculous origin, as additional means and aids for the differential diagnosis of pleural effusions. The necessity for diagnostic aids of this character has been impressed upon the writer since 1913, when he found large numbers of Japanese soldiers and many civilians in Mukden, South Manchuria, with mild pleuritis and exudates of unknown origin. At least 52 per cent of a group of forty-two of these individuals yielded negative v. Pirquet tuberculin skin reactions; cultures of the fluids from twenty-one were negative and tubercle bacilli were not found by smear methods. It is commonly believed that these "light pleurisies" (1) are of tuberculous origin but this has not been proven and the exact etiology is unknown.

Since the demonstration of tubercle bacilli in pleural fluids of tuberculous pleuritis is frequently impossible and no other exact laboratory test being available, studies have been made with the precipitin and complement fixation reactions, and the results are summarized in this article.

MATERIALS AND METHODS

Pleural fluids have been obtained from patients in Philadelphia with tuberculous pleuritis, pneumococcus pleuritis, cardiac insufficiency with pleural transudates and from one case of carcinoma of the pleura with

effusion; also from a large number of guinea pigs and rabbits with experimental tuberculous pleuritis previously described by Kolmer and the writer (2). The latter were particularly useful in this study because means were afforded for determining the approximate time required for the development of complement fixing antibodies in pleural exudates, which was not possible in the human cases of tuberculous pleuritis because of the indefinite histories in relation to the probable duration of infection.

In the majority of instances of experimental tuberculous pleuritis in the guinea pigs, effusions were found in both right and left pleural sacs and frequently in the pericardial and peritoneal spaces; precipitin and complement fixation tests were conducted with all fluids.

Precipitin tests were conducted with pleural fluids as precipitinogens and tuberculosis immune goat and calf sera, kindly furnished by Mr. Glenn, Dr. Paul Lewis and Dr. Aronson. These tests were conducted in the classical manner by laying clear pleural fluids over 0.1 cc. of immune serum in test tubes of appropriate size and reading the results after one and again after twenty-four hours.

The complement fixation tests were conducted according to the technic of Dr. Kolmer's new method for bacterial complement fixation tests (3). Each fluid was first titrated for its anticomplementary activity and employed in $\frac{1}{3}$, $\frac{1}{6}$, $\frac{1}{12}$, $\frac{1}{24}$, $\frac{1}{48}$, etc. of this amount with $\frac{1}{3}$ the anticomplementary unit of an antigen of human tubercle bacilli prepared after a method described by Petroff (4). The primary incubation was eighteen hours in a refrigerator at 6–8°C. plus 10 minutes in a water bath at 38°C., this method having been found by Kolmer to greatly increase the sensitiveness of the tuberculosis complement fixation test over the usual method of water bath incubation for one hour. The secondary incubation was one hour in a water bath, the readings being made a few hours later and recorded after the well known scale of ++++, +++, ++, + and − reactions.

Each human and experimentally produced exudate was also studied bacteriologically, cytologically and the majority for albumin content. The results of these studies with the fluids from experimental lesions are given elsewhere (1); with the fluids from human cases the data was employed with clinical data for establishing the diagnoses.

RESULTS

a. The results of precipitin and complement fixation tests with human tuberculous pleural exudates. These are summarized in

table 1. Of the fluids from twelve cases, five or about 42 per cent yielded positive precipitin reactions; the reactions, however, were quite weak and usually discernible only after twenty-four hours.

All of the fluids but one, however, yielded well defined complement fixation reactions (92 per cent). As shown in this table some of the fluids were hemolytic (nos. 5, 10, 11 and 13) so that the smaller amounts yielded positive reactions while the larger amounts produced partial or complete hemolysis. In every test the exudate controls with all amounts, antigen and hemolytic controls, yielded complete hemolysis and were satisfactory.

Table 2 summarizes the results observed with five pleural exudates of non-tuberculous origin. All yielded negative precipitin reactions; likewise all but one yielded negative complement fixation reactions.

The one fluid reacting weakly positive was from a syphilitic with cardiac decompensation (no. 16); I believe that this result was due to the presence of syphilis "reagin" fixing complement with the lipoids of the tuberculous antigen (5); for this reason the antigen of tubercle bacilli should be freed of lipoids in order to remove the possibility of these cross complement fixation reactions.

b. The results of precipitin and complement fixation tests with pleural, pericardial and peritoneal exudates in experimental tuberculous pleuritis of guinea pigs. These animals were infected with injections of virulent bovine bacilli[1] into the right pleural sacs. Well defined pleuritis was produced in the majority of animals on both sides; in many instances tuberculous pericarditis with effusion also resulted. Tests were made with effusions developing four to twenty-seven days after infection and the results are shown in table 3.

Of eighteen exudates from the right pleural sacs, four or about 22 per cent yielded positive precipitin reactions; eleven or about 61 per cent yielded positive complement fixation reactions. No

[1] Strain H, kindly furnished by Dr. F. Boerner of the Pennsylvania State Livestock Laboratories.

TABLE 1

Results of precipitin and complement fixation with human tuberculous pleural exudates

CASE NUMBER	AGE	SEX	CLINICAL DIAGNOSIS	TUBERCLE BACILLI IN SMEARS	PRECIPITIN REACTIONS	ANTICOMPLEMENTARY UNIT	COMPLEMENT FIXATION REACTION					
							$\frac{1}{3}$*	$\frac{1}{6}$	$\frac{1}{12}$	$\frac{1}{24}$	$\frac{1}{48}$	Control
	years					*cc.**						
1	24	M	T B. pleuritis	None	±	0.1	++	++	+	–	–	–
4	36	M	T B. pleuritis	None	–	0.6	–	–	–	–	–	–
5	7	M	T B. pleuritis	Present	–	0.6	+	+	++	+++	++++	–
6	24	F	T B. pleuritis	None	–	0.6	++++	++++	++++	+++	++	–
7	23	M	Pleuritis (probably T B.)	None	–	0.6	++++	++++	++++	++++	++	–
9	31	F	T B. pleuritis	None	+	0.1	+++	++	+	–	–	–
10	23	M	T B. pleuritis	None	+	0.06	–	–	–	+++	+++	–
11	30	F	T B. pleuritis	None	+	0.06	–	–	++++	++++	++++	–
12	19	M	T B. pleuritis	Present	+	0.1	++	+	±	–	–	–
13	29	M	T B. pleuritis	None	–	0.6	–	–	±	+	+	–
18	27	F	T B. pleuritis	None	–	1.0	++	++	+	–	–	–
20	59	F	T B. pleuritis	None	–	0.06	+	+	–	–	–	–

* $\frac{1}{3}$ of the anticomplementary unit of pleural fluid, etc.

positive reactions were observed with fluids removed earlier than twelve days after infection; after the fifteenth day following infection, nine out of ten fluids or 90 per cent yielded positive complement fixation reactions.

Eighteen left pleural fluids were examined; of these one or 6 per cent yielded positive precipitin reactions but eleven or 61 per cent yielded positive complement fixation reactions. Of nine fluids removed fifteen days or longer after infection of the right pleural cavity, eight or 88 per cent yielded positive complement fixation reactions.

TABLE 2

Results of precipitin and complement fixation tests with human non-tuberculous transudates and exudates

CASE NUMBER	AGE	SEX	CLINICAL DIAGNOSIS	PRECIPITIN REACTION	ANTI-COMPLEMENTARY UNIT	COMPLEMENT FIXATION REACTION					
						$\frac{1}{3}$	$\frac{1}{6}$	$\frac{1}{12}$	$\frac{1}{24}$	$\frac{1}{48}$	Control
	years				*cc.*						
2	47	F	Cardiac insufficiency	–	0.3	–	–	–	–	–	–
3	14	M	Pneumococcus pleuritis	–	0.6	–	–	–	–	–	–
14	adult	M	Pneumococcus pleuritis	–	0.06	–	–	–	–	–	–
16	26	M	Cardiac insufficiency*	–	0.9	+	±	–	–	–	–
17	40	F	Carcinoma of pleurae	–	0.6	–	–	–	–	–	–

* Patient, a syphilitic with aortic insufficiency; blood serum gave positive Wassermann reactions. No evidences of tuberculosis at necropsy.

Two pericardial fluids were examined; both yielded negative precipitin reactions while one (removed eighteen days after infection) yielded strongly positive complement fixation reactions.

Five peritoneal fluids were examined; all yielded negative precipitin and complement fixation reactions. In this connection it may be stated that tubercles were not found in the peritoneum of any of these animals (2), although present in the liver and spleen of a few.

The sera of a number of these animals were also tested and the results observed with nine are shown in table 4. All yielded negative precipitin reactions and five yelded positive comple-

TABLE 3

Results of precipitin and complement fixation tests with pleural, pericardial and peritoneal exudates in experimental tuberculous pleuritis of guinea pigs

ANIMAL	DAYS SINCE INOCULATION	FLUID TESTED	TUBERCLE BACILLI	PRECIPITIN REACTION	ANTICOMPLEMENTARY UNIT	COMPLEMENT FIXATION REACTION							
						$\frac{1}{3}$	$\frac{1}{6}$	$\frac{1}{12}$	$\frac{1}{24}$	$\frac{1}{48}$	$\frac{1}{96}$	$\frac{1}{192}$	Control
					cc.								
Z 5	4	Right pleural	–	–	0.3	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.3	–	–	–	–	–	–	–	–
		Peritoneal	–	–	0.3	–	–	–	–	–	–	–	–
Z 6	8	Right pleural	–	–	0.3	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.3	–	–	–	–	–	–	–	–
		Peritoneal	–	–	0.3	–	–	–	–	–	–	–	–
M 14	10	Right pleural	–	–	0.3	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.3	–	–	–	–	–	–	–	–
M 15	12	Right pleural	+	–	0.3	+	–	–	–	–	–	–	–
		Left pleural	–	–	0.3	+	–	–	–	–	–	–	–
Z 11	12	Right pleural	–	–	0.9	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.9	–	–	–	–	–	–	–	–
		Peritoneal	–	–	0.05	–	–	–	–	–	–	–	–
M 13	13	Left pleural	+	–	0.3	++	+	–	–	–	–	–	–
Z 8	13	Right pleural	–	–	0.5	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.6	±	–	–	–	–	–	–	–
		Pericardial	–	–	0.2	–	–	–	–	–	–	–	–
		Peritoneal	–	–	0.05	–	–	–	–	–	–	–	–
Z 10	14	Right pleural	+	+	0.9	–	–	–	–	–	–	–	–
		Left pleural	–	–	0.9	–	–	–	–	–	–	–	–
Z 12	14	Right pleural	+	±	0.3	+++	++	+	–	–	–	–	–
		Left pleural	+	–	0.3	+++	++	+	–	–	–	–	–

Z 9	15	Right pleural	−	−	0.06	+++	++	−	−	−	−	−	−
		Left pleural	−	−	0.3	+++	++	−	−	−	−	−	−
Z 14	15	Right pleural	−	−	0.3	−	−	−	−	−	−	−	−
		Left pleural	−	−	0.3	−	−	−	−	−	−	−	−
Z 13	16	Right pleural	−	−	0.9	++	+	−	−	−	−	−	−
		Left pleural	−	−	0.6	++	++	−	−	−	−	−	−
G 3	16	Right pleural	−	−	1.0	++++	++++	++++	++++	++++	+++	+	−
		Left pleural	−	−	1.0	++++	++++	++++	++++	++++	+++	+	−
G 6	18	Right pleural	−	+	1.0	++++	++++	++++	++++	++++	+++	++	−
		Pericardial	−	−	1.0	++++	++++	+++	+	−	−	−	−
		Peritoneal	−	−	0.05	−	−	−	−	−	−	−	−
Z 7	21	Right pleural	−	−	0.3	++	+	+	−	−	−	−	−
		Left pleural	−	−	0.3	++	+	−	−	−	−	−	−
G 10	27	Right pleural	+	−	0.3	++	+	−	−	−	−	−	−
		Left pleural	−	−	0.5	++	+	−	−	−	−	−	−
D 3	19	Right pleural	−	−	0.2	+++	+++	++	+	−	−	−	−
		Left pleural	−	−	0.2	+++	+++	++	+	−	−	−	−
D 4	22	Left pleural	−	−	0.3	++++	+++	++	++	+	−	−	−
D 5	22	Right pleural	−	−	0.1	+++	+++	+++	++	+	−	−	−
D 6	25	Right pleural	+	+	0.3	++++	++++	+++	++	+	−	−	−
		Left pleural	+	+	0.3	++++	++++	+++	++	+	−	−	−

ment fixation reactions. One of these animals showed very extensive bilateral pleural, pulmonary and pericardial tuberculosis. All of the sera yielding positive reactions were from animals sixteen days or longer after inoculation.

The sera of thirty control guinea pigs yielded uniformly negative complement fixation reactions.

The results observed with pleural, pericardial and peritoneal fluids from rabbits infected with intrapleural injections of tubercle bacilli, are summarized in table 5. These animals did not

TABLE 4

Results of precipitin and complement fixation tests with the blood sera of guinea pigs with experimental tuberculous pleuritis

ANIMAL	DAYS SINCE INOCULATION	PRECIPITIN REACTION	COMPLEMENT FIXATION REACTIONS					
			0.1	0.05	0.025	0.0125	0.006	Control
Z 5	4	−	−	−	−	−	−	−
Z 6	8	−	−	−	−	−	−	−
M 6	8	−	−	−	−	−	−	−
Z 9	15	−	−	−	−	−	−	−
G 3	16	−	++++	++++	++++	+++	++	−
Z 7	21	−	++	+	±	−	−	−
D 7	26	−	++	+	+	−	−	−
D 8	26	−	+	±	−	−	−	−
D 9	26	−	+++	++	++	−	−	−

show as well marked experimental lesions as the maiority of guinea pigs (2).

Fourteen days or longer after infection the majority of these fluids yielded positive complement fixation reactions and especially the fluids from the right pleural cavities; only one fluid (right pleural cavity) yielded a positive precipitin reaction.

The sera of all animals yielded positive complement fixation reactions, but owing to the well known property of normal rabbit sera for yielding positive non-specific complement fixation reactions, little or no significance can be attached to these results. The reactions observed with the exudates, however, were specific insofar as could be determined.

TABLE 5

*Results of precipitin and complement fixation tests with pleural, pericardial and peritoneal exudates on experimental tuberculous pleuritis**

ANIMAL	DAYS SINCE INOCULATION	EXUDATE	TUBERCLE BACILLI	PRECIPITIN REACTION	ANTI-COMPLEMENTARY UNIT	COMPLEMENT FIXATION REACTION					
						1/3	1/6	1/12	1/24	1/48	Control
					cc.						
R 2	14	Right pleural	+	++	0.05	++++	+++	+	+	+	–
R 3	30	Right pleural	–	–	0.3	+	+	–	–	–	–
		Left pleural	–	–	0.3	+	+	–	–	–	–
		Pericardial	–	–	0.3	+	–	–	–	–	–
		Peritoneal	–	–	0.6	++	–	–	–	–	–
R 9	31	Right pleural	–	–	0.6	+++	+++	++	+	–	–
		Left pleural	–	–	0.6	+++	+++	+++	+	–	–
		Peritoneal	–	–	0.6	++	++	+	–	–	–
R 11	32	Right pleural	–	–	0.3	+++	+++	++	+	–	–
		Peritoneal	0	0	0.03	++	–	–	–	–	–
R 1	35	Left pleural	–	–	0.3	++++	++++	++++	++++	++	–
		Pericardial	–	–	0.3	++++	++++	+++	++	–	–
		Peritoneal	–	–	0.3	++++	++++	++++	++++	++++	–
D 1	22	Pericardial	–	–	0.3	++++	++++	+++	++	+	–
D 2	25	Pericardial	–	–	0.3	++++	++++	+++	++	+	–
D 4	27	Pericardial	–	–	0.15	+	–	–	–	–	–
D 5	27	Left pl. fl.	–	–	0.15	++	+	–	–	–	–
D 8	28	Pericardial	–	–	0.3	+++	++	+	±	–	–
		Peritoneal	–	–	0.15	++	++	–	–	–	–
D 9	28	Pericardial	–	–	0.3	+++	++	+	–	–	–
		Peritoneal	–	–	0.15	+++	++	+	–	–	–

* All of the sera of these rabbits yielded positive tuberculosis complement fixation reactions; all sera yielded negative precipitin reactions.

DISCUSSION

These results indicate that in the exudates from tuberculosis of serous cavities, precipitins and complement fixing antibodies may be found, especially the latter when a sensitive antigen and technic are employed.

In a study of the Wassermann test with exudates and transudates in syphilis, Klauder and Kolmer (6) observed uniformly positive reactions with inflammatory exudates when the reactions with blood sera were negative. Secretions from chancres yielded positive reactions before the syphilis "reagin" could be found in the blood sera indicating a local production of the antibody responsible for the Wassermann reaction.

The results of these studies in syphilis in conjunction with those reported in this paper, indicate that specific antibodies are to be found in the exudates of localized infections at a time when they cannot be demonstrated in the blood sera probably because of their high dilution in the latter. These exudates are readily adapted for complement fixation tests if special attention is given to the following technical steps:

1. The fluid should be centrifuged to remove the cells.
2. The fluid should be heated at 55°C. for fifteen minutes to remove complement, thermolabile hemolysin, thermolabile antilysin, if present, and the substance responsible for the proteotropic reaction. For these purposes longer periods of heating are unnecessary and result in a useless destruction of antibody, as shown by the studies of Kolmer and his associates in syphilis.
3. The fluid should be titrated for anticomplementary activity as they vary greatly in this property and cannot be employed in a fixed amount; similar findings were reported by Klauder and Kolmer in their study of exudates and transudates in syphilis.

Since some fluids are markedly hemolytic it is advisable to use varying amounts beginning with $\frac{1}{2}$ or $\frac{1}{3}$ of the anticomplementary unit as employed in this study. The nature of the hemolytic substance sometimes found in these fluids has not yet been determined.

4. The antigen and complement fixation technic should be as sensitive as consistent with specificity. As previously stated, the antigen employed for tests with human fluids should be free of lipoids as prepared by Kolmer, in order to avoid cross complement fixation tests with tuberculous exudates from syphilitic individuals.

5. It is advisable to employ a control on each amount of exudate tested in order to avoid error with fluids which are hemolytic in large amounts but anticomplementary in smaller amounts.

With these technical considerations it is believed that the complement fixation test will prove of value in the diagnosis of tuberculous pleuritis of fifteen days or longer duration.

CONCLUSIONS

1. Of a series of twelve tuberculous pleural exudates, 42 per cent yielded weakly positive precipitin and 92 per cent well defined positive complement fixation reactions.

2. Human pleural exudates and transudates of non-tuberculous origin yielded uniformly negative precipitin and tuberculosis complement fixation reactions. In syphilis, however, positive reactions may occur due to the presence of the "reagin" in the exudate unless precautions are taken to remove the lipoids from the antigen of tubercle bacilli.

3. With the pleural exudates secured fifteen days or longer after experimental tuberculous pleuritis in guinea pigs, 8 per cent yielded weakly positive precipitin reactions and 89 per cent strongly positive complement fixation reactions.

4. In experimental tuberculous pleuritis and pericarditis of guinea pigs and rabbits, precipitins and complement fixing antibodies are not usually found earlier than twelve days after infection.

5. These results indicate that in the exudates of tuberculous pleuritis, precipitins and especially complement fixing antibodies, are found in a large percentage and that a sensitive complement fixation test with special attention to certain technical features, may prove a valuable practical aid to diagnosis.

REFERENCES

(1) OGAWA, I.: Is pleuritis "army disease?" Igaku Chu-O Zosshi, 1913, 169.
(2) KOLMER, J. A. AND OGAWA, I.: Experimental tuberculous pleuritis in relation to chemotherapeutic studies in tuberculosis. (To be published.)
(3) KOLMER, J. A.: A new complement fixation test for bacterial infections. (To be published.)
(4) PETROFF, S. A.: Glycerin extract of tubercle bacilli as an antigen in complement fixation. Amer. Rev. of Tuberculosis, 1918, **2**, 523.
(5) KOLMER, J. A., AND YAGLE, E.: Bacterial antigens in relation to complement fixation in syphilis with special reference to antigens of tubercle bacilli. (To be published.)
(6) KLAUDER, J. V., AND KOLMER, J. A.: Wassermann test with secretions, transudates and exudates in syphilis. Jour. Amer. Med. Assoc., 1921, **76**, 1635.

ON THE ORIGIN AND NATURE OF ALEXIN (COMPLEMENT) IN GUINEA-PIG BLOOD

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Nomenclature has always played an important rôle in scientific literature. The accepted ethics of terminology recognize and uphold the precedence of priority in cases of dispute providing that the earlier investigator fully describes the substance in question. Owing to individual idiosyncrasies this procedure, in some instances, has been disregarded and serious confusion and controversy has arisen.

Nuttall (1), von Fodor (2) and others recognized and experimentally demonstrated the presence of a bactericidal substance in fresh blood and blood serum but failed to attribute a name to the substance. Buchner (3), in 1889, gave the name of "alexin" to this thermolable, bactericidal, ferment-like, protecting substance. Bordet (4) later verified the presence of this substance and recognized the accuracy of the word "alexin." In addition he discovered the thermostable factor, which also takes an important part in this reaction.

The name alexin was used until Ehrlich and Morgenroth (28) working with the identical, thermolabile property of blood serum some eleven years later, disregarded the existing terminology and described the substance under the name of "complement."

In view of the historical background and the accepted procedure for the adoption of nomenclature it is a singular fact that the standard English texts, with one exception (5) dealing, in part, with the subject of alexin have accepted the later terminology and incorrectly discuss the subject under the heading of "complement." We can only attribute the continuance of the use of this term to the undue precedence given it by the German school.

Apart from the question of priority it is unfortunate that the use of the word "complement" should continue since it indicates an acceptance of the theory of Ehrlich which is certainly relinquishing its hold on the scientific world.

Owing to the fact that the historical and bibliographical background on the subject of alexin has been so ably presented by other investigators only such references as are deemed essential for corroboration or correlation will be incorporated. This paper will take up the subject of the alexin content and the nature of alexin in the blood of normal male guinea-pigs.

I. QUANTITATIVE DETERMINATION OF ALEXIN

Time and temperature relations in clotted and defibrinated blood

Technic. The technic employed in obtaining guinea-pig blood and in titrating its alexin content was in all cases as nearly identical as possible. Normal male guinea-pigs were anaesthetized with ether and the blood obtained by heart puncture. The blood was either allowed to clot, was defibrinated by agitation in a sterile flask containing glass beads or was subjected to other treatment as demanded by the various experiments. In every instance the pooled serum from at least four normal male guinea-pigs and in a few instances as many as forty guinea-pigs was used in order that individual fluctuations in alexin content should not enter into the general considerations. In titrating the alexin content of each sample two series were set up. One series starting at a dilution of 1:2, the other starting at a dilution of 1:10. Subsequent dilutions of these were made in 0.85 per cent NaCl solution, running in multiples of two, resulting in dilutions of 1/2, 1/4, 1/8, 1/10, 1/16, 1/20 and so on to a point well beyond the anticipated titer limit. Intermediate dilutions were not deemed essential as gross differences in alexin content were expected and found. The hemolytic system employed was the usual rabbit serum-anti-sheep cell combination in which three units of the immune serum in 0.5 cc. normal salt solution, 0.5 cc. of a 3 per cent suspension of washed sedimented sheep cells together with varying amounts of reactivating substance in a total volume of 0.5 cc., were employed. After the contents of the tubes were thoroughly mixed, the tubes were incubated for thirty minutes at 37°C. in a water-bath. Readings were made immediately after the tubes were removed from the water-bath and again after they had been

allowed to stand over night in the ice-box. When radical differences in the two readings were encountered the experiment was repeated until check titrations disclosed the cause. The necessary controls were set up for each experiment.

Experiment 1. Normal male guinea-pigs were bled from the heart and the blood of each pig divided into two portions, one of which was defibrinated, the other allowed to clot. As soon as the blood had clotted it was freed from the walls of the container by means of an ordinary nichrome-iron planting wire. After about ten minutes sufficient serum had been squeezed from the clot by contraction of the fibrin net-work to make titration dilutions. The defibrinated blood was centrifugalized for five minutes at 2000 r.p.m. and the supernatant serum pipetted off into a sterile container. Thus we have a quantity of defibrinated blood serum removed from the fibrin clot and cells and a quantity of clotted blood serum which, in this instance, was allowed to remain on the clot until used in the experiment—only sufficient being removed each time to carry on the necessary titrations. Both sera were allowed to remain at room temperature or about 22°C.

An initial titration was made one-half hour after the blood was obtained. Subsequent titrations were made at two-hour-intervals for the first twenty-four hours and at twenty-four-hour-intervals thenceforth until the alexin content of both sera had become negligible.

Titration curves shown in chart 1, plotted from the results of various determinations, show that there is a gross initial difference between the alexin content of the clotted blood serum and the defibrinated blood serum—the former giving a titer of 1 to 10 and the latter a titer of 1 to 64. Following the respective curves one finds that the clotted blood serum rapidly increases in alexin content for the first twenty-four hours and then decreases, at first rapidly, and then less so as time goes on, until at the end of one hundred and forty hours it ceases to be demonstrable. The defibrinated blood serum retains its potency for the first six hours and then rapidly decreases. At the end of twenty-four hours the curve flattens out and the decrease in alexin content is gradual until at the end of one hundred and thirty hours it is negligible. Several repetitions of the above experiment were performed and in no instance were gross deviations from the incorporated curves encountered. In a number of instances the titer of the defibrin-

ated blood sera was found to be much higher—in a few instances giving a titration in a dilution of 1:80—and often the initial titer of the clotted blood serum was as low as 1 to 2. The titration limit of the clotted blood serum never reached the initial titer of the defibrinated blood serum.

Following these experiments, in which the sera were kept at room temperature, a series of experiments were set up so that temperatures from 4°C. to 37°C. could be maintained. The results obtained correspond closely to those obtained by Douglas

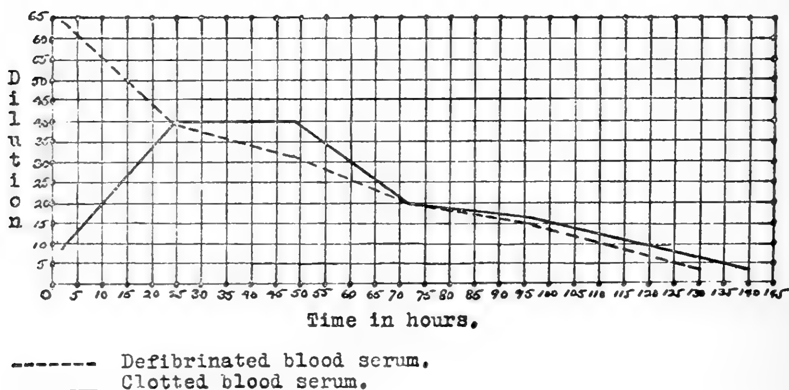

Chart 1. Graphic Representation of the Comparative Titers of Alexin in the Serum of Defibrinated and Clotted Blood in Relation to Time

and Bigger (6), Bigger (7) and Massol and Grysez (8). In their experiments they used serum which had been allowed to remain on the clot for twenty-four hours before being removed and therefore contained the maximum amount of alexin produced by this method. It was found in these experiments, as well as in the former, that the alexin content of the defibrinated blood serum was highest shortly after defibrination and decreased more rapidly at first than it did during the later period of its activity. The differences noticed between the sera kept at ice-box (4°C.) and incubator (37°C.), and those kept at room temperature were that in the first case the process of the decrease in alexin content of

the serum was greatly retarded whereas at 37°C. it was greatly increased.

Attempts to find why the defibrinated blood serum should have an intial alexin titer greater than that ultimately attained by the clotted blood serum allowed to remain on the clot were fruitless. Without going into unnecessary detail some of the main experiments were conducted as follows:

TABLE 1

TIME	A	B	C	D	E	F
hours						
3	1/64	1/64	1/64	1/64	1/64	1/64
24	1/64	1/64	1/64	1/40	1/32	1/40
48	1/40	1/40	1/32	1/32	1/20	1/32
72	1/32	1/32	1/32	1/20	1/20	1/20
96	1/20	1/32	1/20	1/16	1/4	1/20
104	1/10	1/20	*	1/8	1/2	1/10

* Serum contaminated.

TABLE 2

TIME	A	B	C	D	E	F	G	H
hours								
3	Negative	1/40	1/40	1/40	1/40	1/4	1/4	Negative
24	Negative	1/20	1/16	1/20	1/16	1/32	1/16	Negative
48	Negative	1/20	1/16	1/16	1/10	1/20	1/10	Negative
72	Negative	1/16	1/10	1/16	1/8	1/16	1/8	Negative

Experiment 2. Guinea-pigs were bled and the blood defibrinated. The defibrinated blood was treated as follows:

A. Whole defibrinated blood containing serum, red cells, white cells and fibrin clot.

B. Defibrinated blood removed from the fibrin clot.

C. Defibrinated blood serum and red cells. No fibrin.

D. Defibrinated blood serum with white cells, a few red cells and no fibrin.

E. Defibrinated blood serum and fibrin clot. No cells.

F. Defibrinated blood serum alone.

In order to obtain the component parts essential for setting up C, D, E, and F, the defibrinated blood was centrifugalized for ten minutes at 2000 r.p.m. and the supernatant serum divided into four

portions. The white layer of leucocytes was then pipetted off and placed in one of the portions giving (D). The major part of the sedimented red cells was decanted to eliminate as many of the remaining white cells as possible. The remaining cells were mixed with another portion of the defibrinated blood serum giving C. E was set up by the addition of a small piece of the fibrin clot, formed during the process of defibrination, which had the cells removed by washing it in sterile salt solution, to a third portion of the defibrinated blood serum. The results of the titrations are seen in table 1.

After repeating the experiment several times and obtaining similar results it was thought that the leucocytic factor could be controlled by proceeding with the problem in a different manner.

Experiment 3. The leucocytes used in the following experiment were produced by the intraperitoneal injection of extract broth into guinea-pigs and removal of the exudate the following day.

Guinea-pigs were bled and the blood treated as follows:

F. Allowed to clot.

G. Allowed to clot after the addition of leucocytes.

B. Defibrinated, removed from the fibrin clot.

C. Defibrinated after the addition of leucocytes and the blood removed from the fibrin clot.

D. Defibrinated blood serum removed from the cells by centrifugalization.

E. Defibrinated blood serum removed from the cells by centrifugalization with additional leucocytes.

A. *Control.* Leucocytes and salt solution subjected to a similar mechanical agitation as the blood during the process of defibrination.

H. *Control.* Mixture of leucocytes and salt solution unagitated.

In the above experiment the leucocytes were added in quantities of one-tenth volume of the clotted blood, defibrinated blood or defibrinated blood serum, as the case might be, as a suspension in the peritoneal exudate. Owing to the fact that the peritoneal exudate may have acted as the inhibitory element the same protocol was employed with the addition of a control on the possible activity of the peritoneal exudate. The peritoneal exudate containing leucocytes was divided into two equal parts. One part was untreated. The other part was centrifugalized and the supernatant serous exudate decanted. The sedimented leucocytes were washed once with sterile normal salt solution and then resuspended to the former volume in additional salt solution. One series of tubes similar to the preceding protocol were

set up with the untreated peritoneal exudate. Two other series were set up, one in which the supernatant serous exudate was substituted for the untreated peritoneal fluid and the other wherein the suspension of leucocytes in normal saline was used. Titrations gave similar results for all three conditions. Therefore the results tabulated in table 2 were not influenced in any way by the presence of the serous portion of the peritoneal exudate.

It is evident that the leucocytes do not contribute anything to the alexin content of clotted blood, defibrinated blood or defibrinated blood serum, but they act as inhibitory factors on the already present alexin.

One further ramification in our endeavor to locate the seat of alexin production lay in the study of the actual number and integrity of the leucocytes, in conjunction with the respective alexin contents, of two samples of defibrinated blood. One sample of the defibrinated blood to be subjected to ice-box temperature or about 6°C. and the other allowed to remain at room temperature.

Experiment 4. Guinea-pigs were bled, the blood divided into two portions and subjected to the above mentioned temperature conditions. Immediately after the process of defibrination was completed and leucocyte counts had been made, both samples of blood were centrifugalized for five minutes at 2000 r.p.m. and sufficient of the supernatant serum removed for titration purposes. Leucocyte counts and alexin titrations were made almost simultaneously. Subsequent leucocyte counts and titrations were made at intervals of twenty-four hours during a period of one hundred and forty-four hours. After each centrifugalization and removal of a small quantity of serum the sedimented cells and supernatant serum were thoroughly mixed by means of a pipette before the leucocyte counts were made.

The initial titration of the serum of the two samples of blood gave a titer limit of 1/64 and a leucocyte count of 3000 per cubic millimeter of defibrinated blood. At the end of the one hundred and forty-four hour period the blood kept at ice-box temperature gave a serum alexin content titer of 1/16 and a leucocyte count of 2,700 per cubic millimeter of blood whereas the corresponding results for the blood allowed to remain at room temperature were 1/2 and and 2850. The decrease in alexin content showed no anomalies.

During the experiment several smears of each sample were made and stained by Wright's staining method to discover, if present, any structural differences in the appearance of the leucocytes. None were apparent. In the case of the defibrinated blood kept at room temperature the erythrocytes became very fragile during the later portion of the experiment and considerable autolysis was evidenced by the deep red hue taken on by the serum. It is evident that the differences in alexin content of the serum of defibrinated blood as compared with clotted blood when the leucocyte counts are taken into consideration, cannot be explained by the appearance or numbers of leucocytes.

Although there is a marked difference between clotted and defibrinated blood serum at first there is no reason to conclude that this difference is due to the presence or absence of compact fibrin or to the presence, absence, or destruction of either the red or white blood corpuscles.

II. COMPARISON OF THE ALEXIN CONTENTS OF CLOTTED BLOOD AND PLASMA

The work of Gengou (9), later substantiated by Herman (10), apparently demonstrating the absence of alexin as a normal constituent of blood plasma, caused considerable attention to be directed toward the properties of plasma. If this assertion —that plasma does not contain any demonstrable amount of alexin—were true, it would materially assist and substantiate the the claims of the Metchnikoff school in that alexin is a leucocytic product produced in serum after the clotting of blood. However such is not the case. Numerous investigators headed by Domery (11) and running through the recent work of Addis (12) have been able to refute the contentions of Gengou and Herman on a definite and concrete experimental basis. Not only did they repeat the actual experiments of Gengou and obtain diametrically opposite results but, with additional means at their command, conclusively demonstrated the presence of alexin in blood plasma obtained in a number of ways.

For the sake of brevity the results of the titrations of the alexin content of plasma obtained in a number of ways and the necessary control tubes, run simultaneously, are incorporated in a single table which follows the technical and experimental data about to be presented.

After reviewing the literature on the subject of methods for obtaining plasma, three were selected. They were:

Method 1. Salted plasma (Bordet and Gengou, 13)

A sufficient quantity of 20 per cent NaCl solution is added to the whole blood before it clots to give a final concentration of 5 per cent NaCl per total volume of blood and diluent. The salted blood showed no tendency to clot. This salted blood was divided into two main portions, one of which was allowed to remain untreated until needed. The other was centrifugalized and the supernatant salted plasma removed. The salted blood was divided into two portions, 4 parts of distilled water added to each, and one part allowed to clot. The other portion was defibrinated, centrifugalized for ten minutes at 2000 r.p.m. and the supernatant serum removed from the cells. The process of clotting and defibrination took about thirty minutes. The salted plasma was treated in a similar manner except that in the case of the defibrinated plasma—no cells being present—centrifugalization was unnecessary and the plasma serum was decanted from the fibrin clot which had formed around the beads. In all four instances the serum obtained was of a bright red hue showing that the cells had suffered considerably from the effects of the concentrated salt solution.

One point that may prove to be of interest was noticed in the process of defibrination of the salted plasma. When defibrination was started few, if any, bubbles collected on the surface of the fluid but about twenty minutes later, when definite fibrin strands were in evidence, a heavy foam formed and persisted for many hours after the process of defibrination was completed.

Titration of the four types of sera along with diluted and undiluted defibrinated and clotted blood sera were run simultaneously. In the case of the diluted, clotted and defibrinated blood sera the blood, before being defibrinated or allowed to clot, was diluted with 0.85 per cent NaCl solution to a point equal to the final dilution of the salted blood and plasma sera.

Method 2. Oxalated plasma (Watanabe, 14)

Sufficient 1 per cent sodium oxalate solution is added to whole blood so that a final concentration of 0.001 mgm. of the salt per cubic centimeter of blood and diluent is obtained. The oxalated blood was divided into two portions, one of which was centrifugalized for ten minutes at 2000 r.p.m. and the supernatant oxalated plasma removed. Watanabe found that as much as 0.005 mgm. of the sodium salt could be added without interfering with the alexin content of the plasma or of fresh guinea-pig serum to which the salt has been added after the process of clotting has taken place.

The oxalated blood was divided into two portions and sufficient quantities of 1 per cent CaCl-2 solution added to each to give a final concentration of 0.001 mgm. of CaCl-2 per cubic centimeter of oxalated blood and diluent. One part was allowed to clot and the other defibrinated, centrifugalized for ten minutes at 2000 r.p.m. and the supernatant serum removed from the cells. In this manner a crystal clear, almost colorless serum was obtained. The fact that the serum was almost colorless denotes that the cells had suffered a minimal amount of injury.

The oxalated plasma was treated in a manner similar to the oxalated blood. Clotting took place in the oxalated, restored blood and plasma within three minutes after the addition of the CaCl-2 solution.

Titrations of these four sera were run simultaneously with a sample of oxalated, unrestored plasma and normal clotted and defibrinated blood sera as controls.

Owing to the fact that additional substances such as sodium oxalate and calcium chloride had been incorporated into the above experiment it was essential to determine their action, singly and in combination, on guinea-pig serum of known alexin content. Three series were set up, one containing fresh guinea-pig serum with the addition of 1/10 volume of 1 per cent sodium oxalate solution, another with fresh guinea-pig serum with the addition of 1/10 volume of 1 per cent calcium chloride solution, and the third containing 1/10 volumes of both salts added to fresh guinea-pig serum. The guinea-pig serum used throughout was from the same bulk material and therefore of the same alexin content titer. In the first two instances no interference with the alexin content titer was exhibited. In the third instance, wherein a precipitate was formed, a slight decrease in alexin content of the serum was evidenced. This decrease was probably due to the adsorption effected by the precipitate.

Method 3. Paraffin plasma (Gengou, 9)

Conical centrifuge tubes were heavily coated with paraffin, chilled and kept at 0°C. until wanted. Fresh guinea-pig blood was obtained and, before it had clotted, was placed in these tubes and centrifugalized at a low temperature, for five minutes at 2000 r.p.m. The supernatant, fluid plasma was removed by means of a chilled, previously paraffined pipette and divided into two portions, one of which was allowed to clot, the other defibrinated. The plasma serum obtained was crystal clear and colorless. With controls of normal clotted and defibrinated blood sera the alexin contents of the clotted and defibrinated plasma sera were determined.

TABLE 3

Showing the average results obtained in the various experiments dealing with the alexin content of plasma, obtained by the previously described methods, together with the average results of the alexin content determinations of clotted and defibrinated blood, run simultaneously, as controls

TIME	CLOTTED BLOOD SERUM	CLOTTED PLASMA SERUM	DEFIBRINATED BLOOD SERUM	DEFIBRINATED PLASMA SERUM	OXALATED PLASMA
hours					
2	1/10	1/36.1	1/45.3	1/36.8	1/40
24	1/27.5	1/24.4	1/28	1/26.4	1/30
48	1/20.6	1/13.6	1/20	1/18.4	1/20
72	1/17.3	1/10	1/18.6	1/10	1/18
96	1/13	1/9	1/13	1/9	1/12

Before drawing any conclusions from table 3, one point of interest deserves mention. During the preceding experiments with salted blood and plasma considerable dilutions were encountered. This necessitated a corresponding dilution of normal whole blood, prior to its defibrination or clotting, as a control measure and so that the subsequent dilutions in the titrations should be comparable to those of the salted material. In the case of the defibrinated, diluted blood serum nothing extraordinary was noticed. The initial alexin titer of the serum squeezed from the clot of the diluted clotted blood, by the natural contraction of the fibrin strands, was much higher than that of the undiluted clotted blood serum titrated concurrently. This may be explained by the fact that the diluted blood clot contracted much more rapidly than did the undiluted blood clot. The importance of the contraction of

the fibrin net-work will be taken up in detail under the heading of "Discussion."

The results tabulated in table 3 bring out two main points which have been subjects of much discussion. First, they refute the work of Gengou and Herman and corroborate the results obtained by Domery (11) and his successors by conclusively demonstrating the presence of alexin in plasma in equal amounts to that found in the corresponding blood, when allowed to clot, at its highest period of alexin content. And secondly, the demonstration of large amounts of alexin in the plasma, eliminates the fundamental argument of the Metchnikoff school in favor of the leucocytes as the source of alexin in blood serum. In comparing the results obtained with clotted and defibrinated blood plasma and serum one readily admits the advantage of the removal of the fibrin mesh work over the usual methods of allowing it to remain intact.

III. REACTIVATION OF THE ALEXIN CONTENT OF AGED GUINEA-PIG SERUM

On directing our attention to the properties and nature of alexin rather than to its source, it came to our attention that several investigators, working with problems necessitating the use of alexin, had, at some time, found that old guinea-pig serum whose alexin content had "gone bad," owing to the deleterious effects of time and incomplete refrigeration, would regain considerable activity by the addition of relatively small amounts of fresh guinea-pig serum. They found that this "rejuvenated alexin" would fulfill the alexin requirements of their experiments as well as fresh guinea-pig serum which had been allowed to remain on the clot for twenty-four hours prior to its removal and use. This procedure has been followed empirically but not consistently by any one of the investigators. The literature on the subject of alexin makes no note of any such procedure. The nearest approach is the work (15) on the activation of the serum of hereditarily alexin-deficient guinea-pigs by the addition of small amounts of fresh normal serum from various sources or by the addition of such animal fluids as lymph and egg-white. The

subject of the regeneration of alexin in heated serum and serum subjected to the detrimental effects of radiation has been carefully investigated by S. C. Brooks (16) and others.

The suggestion that the addition of equal parts, or less than equal parts of fresh serum to guinea-pig serum whose alexin content has deteriorated will bring the titer of the mixture up to the titer of the fresh serum when titrated alone seemed worthy of investigation.

Experiment 5. A quantity of defibrinated guinea-pig blood serum was obtained and set aside at room temperature until it has lost its alexin content. This process took fourteen days.

Combinations of the old serum and fresh defibrinated guinea-pig serum were set up as follows:

A. Fresh defibrinated blood serum.
B. Old serum.
C. Old serum, 1 part and of fresh serum, 9 parts.
D. Equal parts of old and fresh sera.
E. Old serum, 9 parts and fresh serum, 1 part.
F. Old serum, 9.1 and fresh serum, 0.9 part.
G. Old serum, 9.5 parts and fresh serum, 0.5 part.
H. Old serum, 9.9 parts and fresh serum 0.1 part.

All of the mixtures were set up in separate tubes, thoroughly mixed, and allowed to stand for five minutes before titrations on their respective alexin contents were made.

TABLE 4

Showing the results obtained from the titration of old and fresh serum mixtures

TIME	A	B	C	D	E	F	G	H
minutes								
5	1/80	Negative	1/80	1/80	1/64	1/40	1/32	1/10

From the above data it is found that when equal parts of fresh guinea-pig serum, of high alexin content, are added to old guinea-pig serum, which has lost its alexin content by being allowed to remain at room temperature, the alexin content of the mixture is equal to that of the fresh serum when titrated alone. Assuming

that the old serum acts simply as a diluent one would not expect an alexin content titration greater than 1/40 when the calculations are made on a basis of actual dilution. Table 4 shows a titration of two times this calculated result. When calculated on the same basis the results obtained from the titration of a mixutre of old and fresh serum in proportions of nine to one respectively are far more striking.

Results similar to those presented were obtained when old clotted blood serum was substituted in the place of the old defibrinated blood serum.

The question of the reactivation of serum rapidly inactivated by being subjected to a temperature of 56°C. for one hour presented itself. It was found that this serum was not reactivated by the addition of fresh serum but acted as an inhibitory factor to the alexin present in the fresh serum. Assuming that the heat inactivated serum acted only as a diluent the result of titrating a mixture containing equal parts of inactivated and fresh serum should be equal to one-half that obtained when the fresh serum was titrated. This was not the case. The actual titration received was weakly hemolytic at one-fourth that of the fresh serum. This diminution of the actual alexin content of the fresh guinea-pig serum can be explained on the basis that the larger molecules caused by heating the serum offer themselves as adsorbents.

Taking up the subject of the stability of the reactivated serum mixtures sufficient quantities of the various mixtures were set up in separate tubes so that there would be ample for several titrations.

TABLE 5

Comparison of the stability of the alexin contents of fresh guinea-pig serum and reactivated old sera

TIME	EQUAL PARTS OF OLD SERUM AND FRESH SERUM	OLD SERUM AND FRESH SERUM IN RATIO OF 9:1	FRESH SERUM	OLD SERUM
hours				
½	1/80	1/80	1/80	Negative
24	1/32	1/2	1/40	Negative
48	Negative	Negative	1/32	Negative

From the preceding data it is found that the reactivation process is a transient condition and furthermore that the amount

of reactivation is somewhat dependent upon the quantity of fresh guinea-pig serum used as the activator. The reaction depends, in stability, on the amount of fresh guinea-pig serum incorporated with the old serum. That is, where equal parts of old and fresh serum are used the reactivation is more stable than in the case where only one part of fresh serum was added to nine parts of the old serum although the initial titer of the two be the same.

One additional step in the procedure was incorporated at this point and run simultaneously with the preceding experiment. The cells from the freshly defibrinated blood were recentrifugalized for fifteen minutes at 2000 r.p.m. The supernatant serum and the upper portion of the sedimented cells were pipetted off and discarded, thereby removing the major portion of the serum and white cells present. The remaining cells appeared as a very viscous, dark red fluid. To this lower portion, consisting mainly of red cells, an equal part by volume of the alexin-free, fourteen-day-old serum was added. No mechanical injury was sustained

TABLE 6

SERUM FROM DEFIBRINATED BLOOD FOAM	DEFIBRINATED BLOOD SERUM	SERUM FROM FOAM OF FIBRINATED AGITATED BLOOD SERUM	SERUM FROM FLUID OF AGITATED, DEFIBRINATED BLOOD SERUM
1/64	1/80	1/128	1/64

by the red cells during the process of centrifugalization as there was little or no additional red color given to the serum when it was added to and mixed with the sedimented cells.

In our previous experiments on the reactivation of old serum by the addition of fresh guinea-pig serum we found that the serum, low in alexin content, obtained from clotted blood a short time after it had clotted possessed very little, if any, reactivating property. It was thought that this "aggressive" or reactivating property might be present as an excretion of or extraction from the red cells.

With this object in view titrations of the mixture were run after the mixture had been allowed to remain at room temperature for

one-half hour, twenty-four and forty-eight hours. Respective titrations of 1 to 2, 1 to 2 and negative titrations were obtained. Even these titrations may have been due to the adherence of a small quantity of fresh serum to the cells.

The work Zinsser (5) and others in trying to obtain alexin from leucocytes and the work of Neufeld (17) wherein he demonstrated the absence of alexin within the cell wall of the leucocytes by allowing them to phagocytize highly sensitized red blood corpuscles and observed no intracellular hemolysis or intraphagocytic "shadow forms" is detrimental evidence for the theory of the leucocytic origin of alexin as proposed by many investigators. Considering these facts, together with the data herein presented, it is well within the limits of conservatism to eliminate the blood cells as the source of alexin, at least until evidence, other than that now available, is produced.

IV. THE ENZYMATIC NATURE OF ALEXIN

Solutions of true enzymes, when agitated until a definite foam is produced, show that the foam contains a greater quantity of the enzyme than does the underlying fluid. With this fact in mind it was suggested by Dr. Carl L. A. Schmidt that the foam produced by the rapid agitation of fresh guinea-pig serum might show a higher alexin content and further evidence the generally conceded ferment nature of this substance.

Experiment 6. Guinea-pigs hearts blood was obtained and defibrinated. During the process of defibrination considerable foam formed on the surface of the blood. The defibrinated blood was decanted, centrifugalized at 2000 r.p.m. for ten minutes and the supernatant serum removed. The foam produced during the process of defibrination was pipetted into centrifuge tubes and centrifugalized for a similar period and its alexin content determined. The underlying serum was divided into two portions, one of which was untreated. The other was vigorously agitated for a few moments and the fluid serum decanted from the foam produced. The foam was pipetted into centrifuge tubes and centrifugalized at 2000 r.p.m. for one minute to break up the air bubbles. Titrations of the four types of sera were made one-half hour after the blood was obtained.

Samples of fresh clotted blood serum, of low alexin titer, were subjected to a treatment similar to that of the defibrinated blood serum but no differences in alexin content between the foam and the underlying fluid were recognizable by the method of titration employed.

Experiment 7. The enzymatic nature of alexin was demonstrated by another method.

Sand was thoroughly washed with distilled water, 94 per cent ethyl alcohol and lastly with ether. In this manner it was cleaned, dried and sterilized. Fresh defibrinated guinea-pig blood serum was obtained and divided into two portions. One was allowed to remain at room temperature. The other was added to a quantity of the sand, which had been previously chilled by being allowed to stand in the ice-box overnight, and agitated for five minutes. The chilling of the sand was deemed advisable for two reasons. First, it would retard the adsorption of the alexin and, secondly, it would reduce the detrimental effects of the mechanical agitation to a minimum. Titrations were made one-half, twenty-four and forty-eight hours after the blood was obtained.

TABLE 7

TIME	½ HOUR	24 HOURS	48 HOURS
Unagitated serum	1/80	1/40	1/32
Serum agitated with sand	1/40	1/10	Negative

Under these two conditions it is evident that the alexin in guinea-pig serum reacts like a true enzyme.

The first experiment is of little value other than being corroborative evidence of the enzymatic nature of alexin. The later experiment (exper. 7) is of great importance, as will be shown in the hypothetical explanation of the reason for the difference between the alexin content of fresh defibrinated and clotted blood serum, in that it conclusively demonstrates the possible absorption of alexin by foreign substances.

DISCUSSION

The advantages of the use of defibrinated blood serum over those of clotted blood serum to obtain alexin are many. By the process

of defibrination one is able to obtain a serum, ready for immediate use, with an alexin content almost double that of serum produced in any other manner. This fact offers itself as a great conservation procedure in establishments using large quantities of alexin. The defibrinated blood serum deteriorates in alexin content no more rapidly than does the clotted blood serum.

The disadvantages are few. Owing to the traumatic injury suffered by the cells during the process of defibrination the defibrinated blood serum contains slightly more hemoglobin than does the clotted blood serum, after being allowed to remain on the clot for twenty-four hours prior to its removal.

In reviewing the historical background one finds that defibrinated blood or defibrinated blood serum has been little employed as compared with the mass of experimental data wherein clotted blood serum plays the leading rôle. Von Fodor (2) and Walker (18) worked with the bactericidal value of defibrinated blood serum and blood as compared with clotted blood serum and found that the defibrinated blood and serum were superior. Gurd (19) in working with the variation in alexin content of serum and plasma did use defibrinated blood serum but did not recognise the vast difference between it and the homologous clotted blood serum due to the fact that in defibrinating he agitated the blood for thirty-five minutes at room temperature. By this prolonged agitation at room temperature from one third to one half of the total alexin content procurable was lost. This fact has been clearly demonstrated by the work of Jacoby and Schutze (20), Zeissler (21), Ritz (22) and Noguchi and Bronfenbrenner (23) who worked out the effects, at various temperatures, of mechanical agitation on the alexin content of serum.

Walker (18), in explaining the difference between clotted blood serum and defibrinated blood serum in their action on cultures of *B. typhosus*, lays great stress on the leucocytes as being the source of the bacteriolysin and states that "while in whipped blood the whole available bacteriolysin of the blood is of course available from the onset and undergoes a steady diminution from the first; that of the serum while progressively deterioriating also, is, in the earlier hours, continually receiving fresh additions

from the clot. Accordingly the evidence supports apparently the view that the bacteriolytic 'ferment' is a leucocytic product, and is yielded to the serum by the gradual disintegration of the leucocytes during, and subsequent to the coagulation of the blood." A similar explanation for the increase in alexin of clotted blood serum was sponsored by Gay and Ayer (24) and expresses the generally accepted opinions concerning the origin of alexin as stated by various investigators who have worked on the problem, with the exception of Fassin (25) and possibly Marbé (26) who lay great stress on the thyroid as the source.

The contention of the Metchnikoff school that alexin is of leucocytic origin depends largely upon the findings of Gengou (9), substantiated by Herman (10), that there is no alexin in circulating blood. He came to this conclusion from a series of experiments in which he was apparently able to demonstrate the absence of alexin in clotted plasma serum. However this is not true. Domery (11), followed by a series of investigators, was able to conclusively demonstrate results diametrically opposite to those obtained by Gengou. By repeating the actual experiments of Gengou he was able to demonstrate the presence of alexin in plasma in quantities equal to the greatest amount found in clotted blood serum which had been allowed to remain on the clot. Among the other investigators who were able to refute the contentions of Gengou and simultaneously confirm those of Domery one immediately recognizes the value of the contributions of Watanabe (14) and Addis (12) who not only worked with clotted plasma serum, but, by technical deviations, were able to determine the presence of large quantities of alexin in unclotted plasma, rapidly removed from the supposed sources of alexin with a minimal amount of cell injury.

Why the leucocytic theory of the origin of alexin should hold such prestigé is difficult to explain in view of the absence of substantiating data. Neufeld (17) allowed leucocytes to phagocytize highly sensitized red blood corpuscles and observed that no intraphagocytic hemolysis took place nor were "shadow forms" seen. The digestion of the ingested, highly sensitized red blood corpuscles progressed the same as the digestion of normal unsen-

sitized cells. This conclusively demonstrates the absence of alexin, as one conceives alexin, within the cell wall of the leucocyte. Zinsser (15) allowed living leucocytes to remain, for a considerable period of time, in serum free from alexin and was unable to induce them to produce any demonstrable amount of alexin. All of these facts, together with that portion of this paper wherein the data show that leucocytes were given most advantageous conditions for the production of alexin and not only failed to do so but acted as an inhibitory factor on the already present alexin, present detrimental evidence to the theory that the leucocytes are the source of alexin production.

Heretofore the importance of the extended fibrin network on the alexin content of clotted blood serum has not been recognised. This network presents itself as a large surface to which the alexin adheres. During the first twenty-four hours the fibrin strands contract, pack the cells together and squeeze out the imprisoned serum and alexin by reducing the amount of intracellular space. The tendency of alexin to adhere to extraneous objects has been demonstrated by various investigators, who, in trying to fractionate alexin, passed it through Berkfeld filters and found that the initial portion of the filtrate contained little or no alexin whereas the last portions contained alexin in quantities equal to that found in the serum prior to filtration. Furthermore, in the incorporated experiment, wherein serum of high alexin content was mixed with clean sand, it was found that the sand removed a large portion of the alexin. This adhesion accounts for the low alexin content, during the first few hours, of clotted blood serum from blood which after clotting was carefully freed from the walls of the container and subjected to no further mechanical interference with its natural rate of contraction until sufficient serum had been excluded from the clot for titration purposes. The tube containing the blood clot was then centrifugalized at a low temperature. The serum forced out by this process was found to have an alexin content twenty times greater than that which was exuded by the natural contraction of the clot. Also it is noticed that the clot is greatly contracted.

As evidence in favor of the sponge-like action of the fibrin network of the clot and the lack of activity of the cells we will take into consideration the comparison of the alexin contents of clotted and defibrinated blood serum when studied from a time basis. The temperature relations of the two sera must, necessarily, be kept as a constant factor. Rather than select a single experiment to illustrate the point in question the average results of some twenty experiments, as shown in table 3, columns 1 and 3, will be used.

According to the generally accepted theories the clotted blood serum, when allowed to remain on the clot, receives fresh additions from it in the way of serum and particularily extracts from the degenerating leucocytes during the first twenty-four to thirty-six hours following the clotting of the blood. When the rate of decrease in alexin content of the defibrinated blood serum, removed from all of the supposed sources of alexin by centrifugalization, during the first twenty-four hours and the alexin contents of the two sera, after this first peroid has elapsed, are compared, it becomes evident that the increase in alexin content of the clotted blood serum is not due to leucocytic products. If products of the degenerating leucocytes are added to the serum they are of no help to the alexin content. If they were of significance the alexin content of the clotted blood serum would be, at the end of twenty-four hours, greater than that of the defibrinated blood serum. Such is not the case. The relatively low initial alexin content of the clotted blood serum is due to the adhesion of the alexin to the extended fibrin mesh-work.

Furthermore, histological sections of clots of normal blood made shortly after clotting has taken place and after the clot has been allowed to stand for some time show cell differences in space relation and not structural differences. The cells in the later cases have been considerably distorted due to the pressure exerted by the contracting fibrin strands but there is no visible evidence to show that the cell membrane has been ruptured and the contents forced out. Nor is there sufficient difference in space relation, between the cells of normal circulating blood and those imprisoned within the clot, to lead one to believe that the cell contents have been forced through the membrane.

In the experiments on the reactivation of guinea-pig serum inactivated by being heated to 56°C. for one hour and serum which had been allowed to remain at room temperature until no alexin content was demonstratable, striking differences were obtained. It was found that not only was no reactivation of the heated serum obtained but that it acted as an inhibitory substance. The known alexin content of mixtures of fresh and heated serum was reduced to a point lower than could be accounted for by the actual dilutions made. During the heating process of the inactivated serum the molecular structure of the serum is changed, and aggregation of larger molecules resulting. The reduction of the alexin content of the fresh serum, added to the heated serum, past the point of actual dilution is due to the adsorption of the alexin by these larger molecules.

The inactivation of the alexin content of fresh guinea-pig serum by continuous agitation, as carried out by Noguchi and Bronfenbrenner (23) and their predecessors (20, 21, 22,) may be explained by the same mechanism of molecular change.

With the unheated serum, allowed to remain at room temperature until it had lost its alexin content, a different state of affairs was demonstrated. By the addition of a relatively small amount of fresh serum, namely, 10 per cent, to the old serum which had no alexin content, the alexin content of the mixture was brought up to the same height as that of the fresh serum when titrated alone. This reactivated serum alexin is not as stable as that of the fresh alexin. The stability is directly dependent upon the relative amounts of fresh and old serum.

The works of Brand (27) Ferrata and their contemporaries, on the dual nature of alexin should not be confused with the explanation about to be presented. This reactivation of guinea-pig serum, allowed to remain at room temperature until it has lost its titratable alexin, may be explained on the basis that there are two thermolabile portions of alexin. One fraction is more stable than the other and is unable, of itself, to react with the immune serum-sheep cell combination to bring about hemolysis of the sensitized sheep cells. The less stable fraction, found in large quantities in fresh defibrinated blood and clotted blood serum

which has been allowed to remain on the clot for twenty-four hours prior to its removal and use, is capable of uniting with a relatively large amount of the more stable factor, present in the old serum, and to reactivate it.

If this idea of alexin being composed of two parts, one more thermolabile than the other, is not tenable, as further work on the subject may show, the reactivation of the old serum may be accounted for by the enzymatic nature of alexin in that it is, under favorable conditions and in the absence of inhibiting factors, capable of being active in concentrations less than the calculated titer limit.

The enzymatic nature of alexin is demonstrated by comparing the action of guinea-pig serum, high in alexin content, with the generally accepted reactions of pure enzymes. First, by agitating the serum for a short time, producing a foam, and demonstrating that the foam has a greater alexin content than the fluid beneath it. Secondly, by the adsorption of alexin by foreign bodies, offering a large contact surface. In one instance, sand was used to demonstrate this adsorption. And thirdly, by its capability, under favorable circumstances, to act in dilutions greater than the calculated titer limit.

CONCLUSIONS

A far more powerful alexin, or complement, ready for immediate use, is obtained by the defibrination, centrifugalization and removal from the cells of guinea-pig blood serum than is obtained by the usual method of allowing the blood to clot and removing the serum after it has been allowed to stand on the clot for twenty-four hours.

The same time and temperature relations, as have been worked out for clotted blood serum alexin, hold true for the defibrinated blood serum alexin content.

The leucocytic theory of the origin of alexin is not tenable when the data detrimental to such a theory, now available, are taken into consideration. The experimental evidence presented showing the apparent inactivity of either the red or white blood corpuscles to produce alexin, together with the fact that blood

plasma contains large amounts of alexin, offer themselves as strong arguments against such a theory.

There is a gross difference between serum inactivated by heating to 56°C. for one hour and serum allowed to stand at room temperature until it has lost its alexin content, in their reactibility on the addition of fresh guinea-pig serum, high in alexin content. This difference is due to the adsorption of the alexin of the fresh serum by the larger molecules of the heated serum. The reactivation of the old serum may be explained by the enzymatic nature of alexin or by assuming that there are two thermolabile fractions of alexin, one being more susceptible to the detrimental effects of time and temperature than the other.

Fresh guinea-pig serum allowed to stand at room temperatures for several days showed no further evidence of "complementoids."

Further data in corroboration of the enzymatic nature of alexin, were obtained.

REFERENCES

(1) Nuttall, G.: Zeit. f. Hyg. 1888, **4**, 353.
(2) von Fodor, J.: Centralbl. f. Bakt., 1890, **7**, 753.
(3) Buchner, H.: Centralbl. f. Bakt., 1889, **5**, 817; **6**, 1.
(4) Bordet, J.: Ann. de l'Inst. Past., 1899, **13**, 225, 273.
(5) Zinsser: Infection and Resistance. (Macmillan & Co., New York, 1914.) Chapter VII, p. 168.
(6) Douglas, J. S. C., and Bigger, J. W.: Lancet, London, 1918, **2**, 44.
(7) Bigger, J. W.: Jour. Path. and Bact., 1919, **22**, 323.
(8) Massol, L., and Grysez, V.: Comp. rend. Soc. de Biol., 1910, **68**, 825.
(9) Gengou, O.: Ann. de l'Inst. Past., 1901, **15**, 232.
(10) Herman, M.: Bull. de l'Acad. Roy. de Med., 1904, 4 ser. **18**, 137.
(11) Domery, P.: Wien. Klin. Wochenschr., 1902, **15**, 1025.
(12) Addis, T.: Jour. Inf. Dis,. 1912, **10**, 200.
(13) Bordet, J., and Gengou, O.: Ann. de l'Inst. Past., 1903, **17**, 822; Ann. de l'Inst. Past., 1904, **18**, 98.
(14) Watanabe, S.: Jour. Imm., 1919, **4**, 77.
(15) Ecker, E. E.: Jour. Inf. Dis., 1921, **29**, 611.
(16) Brooks, S. C.: Jour. Med. Resch., 1920, **41**, 411.
(17) Neufeld, F.: Arb. a. d. Kais. Gesundheitsamt., 1908, **28**, 125.
(18) Walker, E. W. A.: Jour. Hyg., 1903, **3**, 52.
(19) Gurd, F. B.: Jour. Inf. Dis., 1912, **11**, 225.
(20) Jacoby, M., and Schutze, A.: Zeit. f. Imm., 1910, **4**, 730.
(21) Zeissler, J.: Berlin Klin. Wochenschr., 1909, **46**, 1968.
(22) Ritz, H.: Zeit. f. Imm., 1912. Orig., **15**, 145.
(23) Noguchi, H., and Bronfenbrenner, J.: Jour. Exp. Med., 1911, **13**, 229.

(24) Gay, F. P., and Ayer, J. B.: Jour. Med. Resch., 1907, **17**, 341, 361.
(25) Fassin, L.: Comp. rend. Soc. de Biol., 1907, **62**, 647.
(26) Marbé, S.: Comp. rend. Soc. de Biol., 1908–1909, **64**, 1058, 1113; **65**, 612; **66**, 432, 1073; **67**, 54, et seq.; **68**, 882, et seq.
(27) Brand: Berlin Klin. Wochenschr., 1907, **34**, 320.
(28) Ehrlich, P., and Morgenroth, J.: Berlin Klin. Wochenschr., 1900, **37**, 453, 681.

A STUDY OF THE HEMOLYTIC ANTIBODY-ANTIGEN COMBINATION

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I. INTRODUCTION

According to Ehrlich (1), a formed antigen, such as a red cell or bacterium, has a definite number of combining groups or bonds which have an affinity for the specific antibody. When the antigen is placed in contact with its antibody, a reaction ensues, which progresses until all these bonds are satisfied or until all the antibodies have entered into the combination. It follows from this that when all the combining affinities of the antigen are satisfied, no more antibodies can enter into the reaction regardless of the number that still remain free in the supernatant fluid. This theory implies chemical affinities and a combination according to the respective valencies of the reacting substances.

The view of Arrhenius was somewhat opposed to this (2). He regarded the reaction as a physical one and explained the phenomenon as a distribution of a solute between two solvents, the antibodies being the solute, and cell protoplasm and the surrounding fluid the two solvents. He states that "the immune bodies are probably not bound by the erythrocytes, but only absorbed by them," and that "no proof has been given of their chemical action."

Bordet's idea (3), although not the same as that of Arrhenius, was similar in that he attempted to explain the phenomeonon according to physical laws. He, however, held that the antibody or "sensitizing substance" is adsorbed in much the same way that a filter paper takes up a dye. He states that "the union of the antibody with the antigen depends on what is called molecular adhesion or contact affinity, in other words, should be classed in the category of adsorption phenomena."

It has long been known that a red cell or bacterium will combine with much more than the amount of antibody necessary for lysis. Arrhenius (2) found that this varies with the concentration, and that a red cell will take up several thousand units, if the concentration of the antibodies in the supernatant liquid is sufficiently high.

Arrhenius expressed the reaction according to the equation,

$$B = KC^n$$

in which B represents the amount of antibody absorbed by the cells, C the concentration remaining in the supernatant liquid after absorption, and K and n are constants. He found the value of n to be $\frac{2}{3}$, and from the equation and the calculated value of n, he interpreted the reaction as a distribution of the antibodies between the red cell protoplasm and the surrounding liquid as solvents, and that two of the antibody molecules free in the serum form three of the combined molecules.

Manwarning's work (4, 5, 6, 7, 8) on this same phenomenon failed to confirm the conclusions of Arrhenius. He found that the absorption did not follow any simple physico-chemical law, and that K and n were not constants. Manwaring obtained what he calls a "negative absorption," i.e., the titer of the serum dilutions, when great concentrations of antibody units were used, was often greater after contact with the corpuscles than before. He concluded, therefore, that qualitative changes take place in the amboceptor due to its contact with the cells, and "that any direct quantitative comparison between it and the untreated serum gives erroneous results."

From these and other experiments, he came to the conclusion that there is a "third component" in the serum besides the antibody and complement, which varies in quantity in different animals. This third component may be "antilytic," or "auxilytic," but never has independent hemolytic powers, although it may be absorbed by the red cells. In addition, he mentioned several other factors influencing the antigen-antibody combination, among which are the reaction and specific gravity of the medium and the amount of inorganic salts.

Amato (23), in a recent study of opsonic sensitization of bacteria, has come to the conclusion that the union of the opsonins

with the bacteria is governed by the same law that governs the union of hemolysins with the antigen. He found the same equation applicable to the opsonins which Arrhenius applied to the hemolysins and concluded that the reaction is probably a distribution of the opsonins between two solvents in which they have different molecular weights.

Coulter (9, 10) has done some very definite work on the influence of the reaction of the medium on the absorption of hemolytic sensitizer by red cells, and on the dissociation of the combination. He found pH = 5.3 to be the optimum H-ion concentration for the absorption. However, he also found very little difference in absorption between the values, pH = 4.5 and pH = 6.0, in the salt-free medium, and a much wider range in medium containing salt.

Kahn (11), in a very recent work on the absorption of hemolytic sensitizer, has made some studies on the rate of the reaction at the various temperatures. He finds that the reaction is completed very quickly, in every case at the end of fifteen minutes. He also finds "that the extraction is greater at 37° than at room temperature, which in turn is greater than at ice-box temperature."

It is seen, then, that the antigen-antibody combination is influenced by time, temperature, the reaction of the medium, and the amount of inorganic salts. In addition, there is also considerable variation due to uncontrolled factors, which vary with the different lots of serum used—those variations which caused the differences of opinion between Arrhenius and Manwaring.

In the face of all that has been done on the absorption of hemolytic antibodies by red cells, it would seem almost hopeless to try to add anything new, either in fact or theory. However, it is thought that the results of the experiments recorded in this paper are significant in suggesting an explanation of the differences obtained by Arrhenius and Manwaring, without the necessity of resorting to the sub-divided "third component," or to any qualitative changes in the amboceptor due to its contact with the red cells.

In all the discussions the terms, "amboceptor," "antibody," "sensitizer," and "immune body," will be used interchangeably.

II. THE CONCENTRATION OF THE HEMOLYTIC ANTIBODIES AS A FACTOR INFLUENCING THEIR ABSORPTION BY RED CELLS

These experiments were carried out in order to determine how many times the amount of sensitizer necessary for hemolysis the red cell will absorb, and whether or not there is a definite saturation point, as might be assumed from Ehrlich's theory, above which the red cells will absorb no more, regardless of the amount still remaining free in the serum.

The technic was as follows: Rabbits and guinea-pigs were immunized to the red cells of the sheep, by intraperitoneal injections of the guinea-pigs and intravenous injections of the rabbits. The serum so obtained was inactivated at 56°C. for thirty minutes and then carefully titrated against fresh sheep cells that had been collected in 1 per cent sodium citrate and washed four times in large volumes of normal salt solution. The unit of cells was arbitrarily chosen as 0.1 cc. of 1:4 suspension[1] (measured in terms of whole blood), and the unit of complement as 0.1 cc. of a dilution of fresh normal guinea-pig serum, pooled from several animals. The unit of antibody or sensitizer was defined as the smallest amount of the immune serum which, under the above conditions, would just suffice to hemolyze a unit of sheep corpuscles in a total volume of 1 cc., in one hour's incubation at 37°C. Protocol 1 will illustrate.

Protocol 1. Type for hemolytic titrations

	TUBES								
	1	2	3	4	5	6	7	8	9
Immune serum* 1:200	0.5	0.4	0.3	0.25					
Immune serum* 1:500					0.5	0.4	0.3	0	0
Guinea-pig complement 1:5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0
Sheep cells 1:4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt solution	0.3	0.4	0.5	0.55	0.3	0.4	0.5	0.8	0.9
Hemolysis one hour†	4+	4+	4+	4+	4+	3+	2+	0	0

* The numbers, 1: 200 and 1: 500, represent the dilutions of the immune serum used in the tubes. For instance, tubes 1 to 4 received 0.5, 0.4, 0.3, and 0.25 cc. of a 1:200 dilution, respectively.

† 4+ means complete hemolysis. The degrees of hemolysis are indicated by the signs, 3+, 2+, and +.

[1] All dilutions and suspensions were made in normal salt solution. A 1:4 suspension of cells means one part of cells plus three parts of salt solution.

In the above sample titration, it is seen that the hemolytic unit of this particular serum is 0.5 cc. of a 1:500 dilution, or 0.001 cc. Then 1 cc. of this serum contains 1000 hemolytic units. The titer is expressed as 1/1000, or sometimes as 1000.

In making the absorption tests, a number of tubes were arranged, each containing two units of sheep cells and sensitizer in varying amounts. Enough salt solution was added to each tube before the sensitizer to make the final volume 2 cc. The tubes were incubated in a water bath for thirty minutes at 37°C., after which the red cells were removed by centrifugalization and the supernatant fluid titrated as in protocol 1, to determine the number of units of sensitizer lost, i.e., the number removed by the red cells. The use of all the materials concerned in the reaction in only twice the amount ordinarily used for titration overcame the necessity for the use of an undue amount of immune serum and still afforded sufficient supernatant fluid for the subsequent titration. The method is shown in protocol 2.

*Protocol 2. Type for absorption of hemolytic units by sheep red cells**

TUBE NUMBER	SENSITIZER		SHEEP CELLS 1/4	SALT SOLUTION	TITER AFTER ABSORPTION	UNITS ABSORBED
	Units per cubic centimeter	Quantity of serum				
		cc.				
1	5	1.0 (1:400)	0.2	0.8	0	5
2	10	0.2 (1:40)	0.2	0.6	0	10
3	20	0.4 (1:40)	0.2	1.4	0	20
4	50	1.0 (1:40)	0.2	0.8	1/2	48
5	70	1.4 (1:40)	0.2	0.4	1/8	62
6	100	0.2 (1:4)	0.2	1.6	1/12	88
7	200	0.4 (1:4)	0.2	1.4	1/40	160
8	500	1.0 (1:4)	0.2	0.8	1/150	350
9	1,000	0.5 (undiluted)	0.2	1.3	1/400	600
10	2,000	1.0 (undiluted)	0.2	0.8	1/1000	1000
11†	50	1.0 (1:40)	0	1.0	1/50	0
12	500	1.0 (1:4)	0	1.0	1/500	0

* All the sets were made in duplicate.

† Sets 11 and 12 are controls.

Tables 1 and 2 show the absorption from immune rabbit and guinea-pig serum in concentrations ranging from 5 to 2000 units per cubic centimeter. On the first line of each table are shown the concentrations with which the cells were treated, and on the

following lines are shown the amounts of antibody taken up by the cells from each concentration in the tests made with the different lots of serum. Some of the results are also shown graphically in figures 1, 2, and 3, in which are plotted the logarithms of the

TABLE 1

Absorption of antibodies from rabbit immune serum by sheep erythrocytes

		Concentration of antibody units per cc. in the serum dilutions							Remarks
		20	50	100	200	500	1000	2000	
Units absorbed	Test 1	20	49	90	180				
	Test 2	20	48	92	175	340			Titer 1/10,000
	Test 3	20	47	86	160	300	360		Serum of test 2, stored four months, titer 1/6000
	Test 4		47	90	167	375	500	670	
	Test 5		47	92	150	375	500		
	Test 6	19	47	88	150	300	500	750	
	Test 7	18	30	44	77				Titer 1/1000
	Test 8	20	49	97	180	300			
	Test 9		49	95	193	450	700	1200	Titer 1/40,000
	Test 10		49	98	185	350	540	875	Serum from same rabbit as in test 9, eight days later. Titer, 1/25,000
	Test 11		47	87	150				

TABLE 2

Absorption of hemolytic antibodies from immune guinea-pig serum by sheep erythrocytes*

		Concentration of antibody units per cc. in the serum dilutions							Titer
		5	10	20	50	100	200	500	
Units absorbed	Test 1†	5	10	19	41	70	100		1/4000
	Test 2	4	7	12	20	40	70		1/2500
	Test 3	4	6	12	25	40	70	235	1/1000
	Test 4	4	6	10	20	33	40	100	1/1000
	Test 5	4	5	8		50			1/1000
	Test 6	3		4	10	20			1/125
	Test 7				17	20	33		1/2000

* From 1000 units sensitizer from guinea-pig serum the absorption was so often difficult to determine that it is not listed here. Often no difference in titer could be detected after contact with the cells.

† Serum of test 1, stored four months.

amounts absorbed against the logarithms of the concentrations remaining in the liquid after the absorption is completed. The plot, as is seen, tends to approach a straight line.

A study of tables 1 and 2 brings out several important facts. Even from comparatively low concentrations of antibody units all are not absorbed, while, if the concentration in the liquid is sufficiently high, massive quantities are taken up. The only

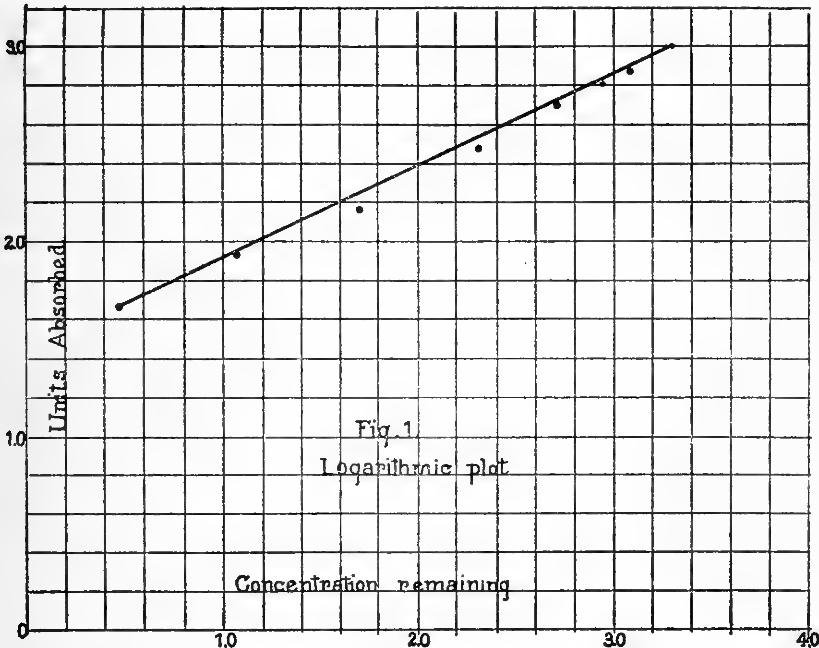

FIG. 1. LOGARITHMIC PLOT OF THE DATA OF TABLE 3

The amount of sensitizer absorbed is plotted against the concentration remaining in the supernatant liquid. Titer of serum, 1/4,000. $K = 22.18$, $n = 0.5$

instances in which the reaction seems to be complete are when very low concentrations are used, and even here, the assumption that all the sensitizer is absorbed does not seem justified, because amounts much less than one unit per cubic centimeter could not be detected by the method of titration used in these experiments. It is evident, therefore, that the number of amboceptor units taken up by the sheep red cells varies with the concentration of

those units in the surrounding medium; the more concentrated the units are, the more are absorbed.

Another fact to be noted is that the absorption is nearly always higher from the rabbit serum than from the guinea-pig serum,

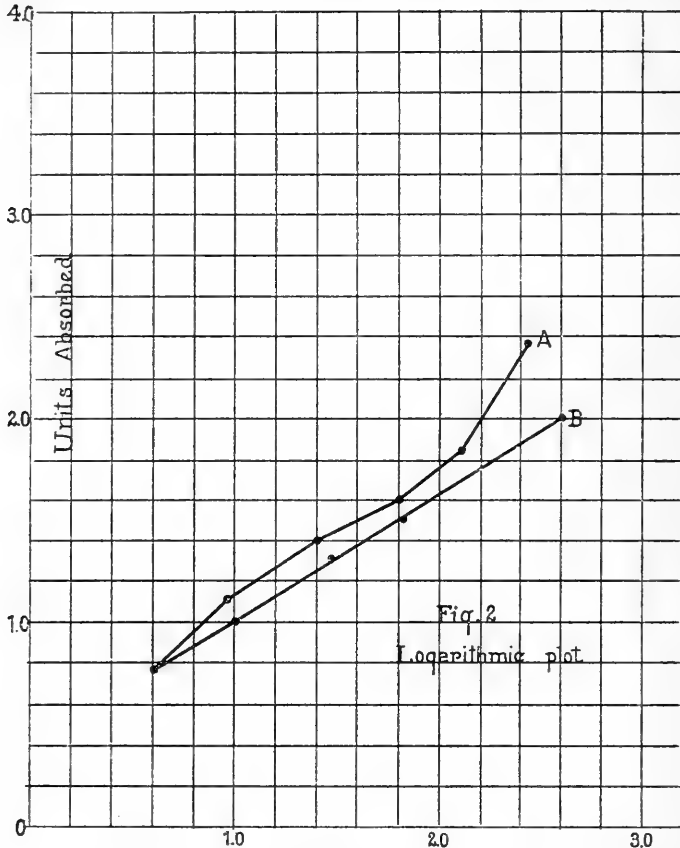

FIG. 2. CURVES A AND B, LOGARITHMIC PLOTS OF THE DATA OF TESTS 3 AND 4 OF TABLE 2

The data for these plots are also shown in table 6. For curve A, $K = 3.225$, $n = 0.78$.[4] For curve B, $K = 1.97$, $n = 0.61$.

and that the absorption varies with the serum of individuals of the same species. This will be referred to later.

The results here recorded agree, in part, with those obtained by Arrhenius and Morganroth (2) in their work on the absorption

of immune bodies by ox and sheep cells, in which they were able to express the reaction according to the equation,

$$B = KC^n$$

This equation holds true, however, only when the logarithmic plot, as shown in the figures, represents a straight line; and the farther it deviates from the straight line, the more inapplicable

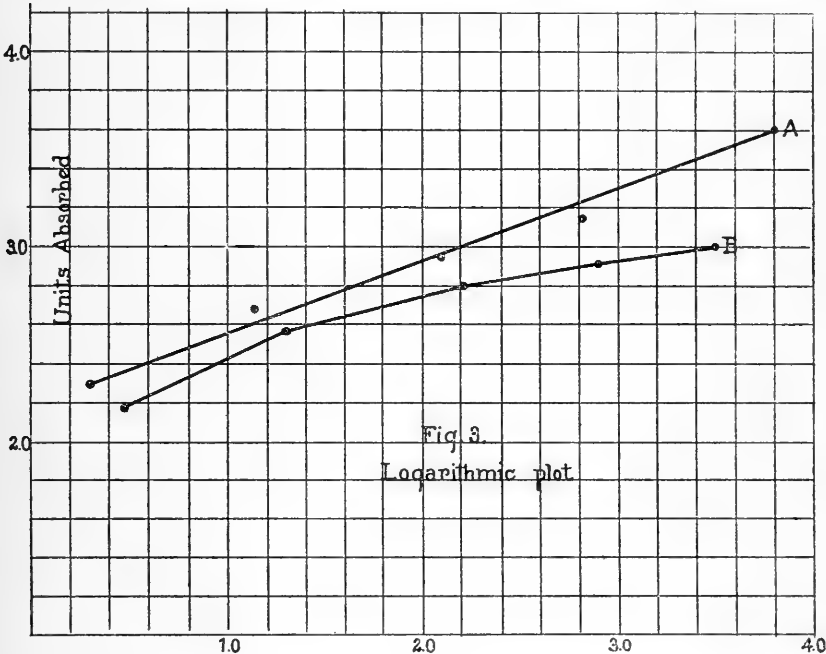

FIG. 3. LOGARITHMIC PLOTS, A AND B, REPRESENTING THE DATA OF TABLES 4 AND 5

The sera were obtained from rabbit 69 on the tenth and twenty-fourth days, respectively. For curve A, $K = 165.9$, $n = 0.36$. For curve B, $K = 80.73$, $n = 0.4$.

becomes the equation, because of the variation in the values of the constants, K and n, between the different concentrations. The tests represented by figs. 1 and 3A show practically straight line plots, so here the equation can be applied. The experimental and calculated results compare very favorably, as is shown in tables 3 and 4, which correspond to figures 1 and 3A, respectively.

The disagreement is, in most cases, within the limits of experimental error. The values of the constants, K and n, should be

TABLE 3

Experimental and calculated results from test 6, of table 1

B*	C observed	C calculated	K	n
46	4	4.4	22.18	0.5
88	12	15.7		
150	50	45.7		
300	200	170.3		
500	500	508.2		
750	1250	1144.0		
1000	2000	2034.0		

* B = the number of units absorbed by the cells, C = the concentration remaining after absorption.

TABLE 4

Experimental and calculated results from absorption tests made on the serum of rabbit 69, obtained on the tenth day

B	C observed	C calculated	K	n
198	2	2	165.9	0.36
487	13	24		
875	125	124		
1340	660	408		
2350	1650	1950		
4000	6000	8000		

TABLE 5

Results of absorption tests on the serum of rabbit 69, obtained on the twenty-fourth day

B	C observed	C calculated	K	n
157	3	5	80.73	0.4
380	20	15		
630	170	170		
800	800	309		
1000	3000	540		

noted. The "absorption constant," K, is 22.18 for table 3, and for table 4, $K = 169.5$, which is very much higher. The absorption is also very much higher from all concentrations in table 4.

The value of n differs for the two tables, and in both it is very much lower than the value, $\frac{2}{3}$, which was found by Arrhenius. In figure 1, n = 0.5, and in figure 3A, $n = 0.36$; i.e., the curve having the steeper gradient has also the higher value for n. Thus, it is seen that the constants vary with the different lots of serum used, the value of K, determining the position of the curve, and of n, the gradient.

TABLE 6

Experimental and calculated results from absorption of sensitizer from guinea-pig immune serum by sheep erythrocytes

B	C OBSERVED	C CALCULATED	K	n
Curve A, figure 2				
6	4	2.54	3.225	0.78
12	8	7.19		
25	25	21.52		
40	60	43.68		
70	130	101.20		
235	165	622.10		
Curve B, figure 2				
6	4	5.3	1.969	0.61
10	10	11.5		
20	30	32.8		
33	67	68.9		
40	160	91.6		
100	400	362.3		

In figure 2, there are shown two plots, A and B, representing the two tests shown in table 6. Curve A deviates considerably from the straight line so the values, $K = 3.225$, and $n = 0.78$, are necessarily calculated only approximately from the averages of several determinations made at intervals along the curve. There is also in the table quite a discrepancy between the observed and calculated results. The table for curve B, which approximates the straight line very closely, shows a very close agreement between the observed and the calculated results.

III. THE RATE OF THE REACTION BETWEEN THE ANTIGEN AND ANTIBODY, IN VITRO

It would seem that the union of the antigen and antibody is extremely rapid, and practically instantaneous in vivo. This is demonstrated by the anaphalactic shock in hypersensitive animals, for when the antigen is injected into the blood-stream, the shock often occurs immediately. Bull (12) has found pneumococci, when injected into the blood-stream of an immune animal, to be agglutinated within a very few seconds.

In the previous absorption experiments, the cells were allowed to remain in contact with the sensitizing serum for thirty minutes, which was considered sufficient time for the reaction to reach equilibrium. In order to control this, however, it was necessary to establish definitely whether or not the time allowed was enough, and how much it could be varied without influencing the results.

TABLE 7

Rate of the antigen-antibody reaction

	CONCENTRATION OF ANTIBODY UNITS PER CC. IN THE SERUM DILUTIONS						TIME
	100	100	165	250	500	2000	
							minutes
Units absorbed		60	65	80	250		1
	84	82	100	120	350	400	5
	87	88	100	125	375	400	15
	87	88	100	120	375	400	30
	90	90	100	125	375	400	60
	87	88	100	125	375	400	120

In this experiment, sheep cells and sensitizer were allowed to remain in contact for varying periods of time and the amount taken up tested by titration, as in the previous experiments. The longest time was two hours. The shortest time, which is listed as one minute in the table, could not be kept absolutely constant. The corpuscles were added to the serum dilutions, shaken up thoroughly, and immediately centrifuged at high speed. The time during which the cells were in free contact with the serum was certainly not more than two minutes in any case. The results are given in table 7.

It is evident, from this table, that equilibrium is reached very quickly. It is practically complete within five minutes, and entirely so in every case at the end of fifteen minutes. A slight dissociation, at the end of two hours, is indicated in the absorption from 100 units, but this is not very pronounced and does not show at all in the higher concentrations. The data given here are few, but the experiment serves its purpose as a control to the other experiments, and shows that the time allowed for the absorption may be varied within a wide range without materially influencing the results.

IV. THE INFLUENCE OF TEMPERATURE ON ABSORPTION OF ANTIBODIES BY RED CELLS

A temperature of 37°C. is employed in most serological reactions. The combination of the antigen and antibody, however, takes place at much higher and much lower temperatures. Differences of opinion occur among the different investigators as to the best temperature for the combination. Neil (13) and Kolmer (15) advise the use of room temperature as the best for the sensitization of cells, while Hinton (14) recommends 37°C. These opinions were advanced as the result of more or less extensive work on the standardization of the Wassermann reaction. Kahn (11) states that "the extraction is greater at 37° than at room temperature, which in turn is greater that at ice-box temperature."

In the experiments here recorded, the influence of the variation of the temperature upon the absorption of hemolytic antibodies has been observed. The absorption tests were carried out as in the foregoing experiments, using the rabbit anti-sheep hemolytic system. Duplicate sets were made for each temperature and concentration tested. The antibody dilutions were made first, and the cells added after both had been brought to the required temperature. It is needless to say that the tests at all different temperatures with one lot of serum were made on the same day with the same lot of cells and complement. The results are given in table 8.

TABLE 8

The influence of temperature upon the antigen-antibody reaction

(A)

	Concentration of antibody units per cc. in the serum dilutions						Temperature
	Series A Serum	Series B Serum	Series C Serum			Series D Serum	
	100	600	65	130	320	400	
							°C
Units absorbed	92	325	55	105	120	108	0
	95	475	60	120	190	175	15
	96	475	62	120	200	180	25
	97	475	62	130	220	220	37
		500					40
		475					45
		440					50
		400					55

(B) Serum of rabbit 487

	Concentration of antibody units per cc. in the serum dilutions						Temperature
	100	100	200	200	500	800	
							°C
Units absorbed	92	93	180	178	330	580	0
	95	95	187	183	340	600	15
	95	95	187	184	340	600	25
	90	93	180	178	330	575	37
	88		175	167		575	40
	83		163	160	320	550	45
	75		155	150	310	450	50
			120		300	400	55
			75			300	60

(C) Absorption from 100 units of sensitizer from the sera, E to I

Absorption from	Temperature			
	0°	Room	37°	45°
Sera E	88	94	95	90
Sera F	93	96	94	87
Sera G	100	100	100	99
Sera H	40	50	55	45
Sera I	50	70	60	50

TABLE 8—*Continued*

(D) Absorption from 200 units of sensitizer from the sera, E to I

ABSORPTION FROM	TEMPERATURE			
	0°	Room	37°	45°
Sera E	145	170	185	175
Sera F	167	183	170	155
Sera G	199	200	200	199
Sera H	120	160	165	135
Sera I	80	120	100	100

In the tests with the sera A, B, C, and D the absorption was greater at 37° than at the lower temperatures. That is, the absorption at 37° >25° = or >15° >0°. In the tests with the serum of rabbit B, the absorption was greater at 40° than at any other temperature.

The serum from rabbit 487, however, when tested gave a different result. Seven tests were made on this one lot of serum at different times, using the various concentrations listed in the table. In every test, the temperature of 15° to 25° proved to be the optimum, lower absorptions being obtained at 0°, and 37°, and above. The relative amounts of absorption for this serum may be expressed thus: 15° = 25° > 0° = or > 37°. It is true that the differences in absorption between 15 and 37° are not great, but they consistently appear in all the concentrations tested.

The results obtained with this serum suggested that the optimum temperature for the absorption might be a variable, which is different for the different sera. Therefore, other lots of serum, which had been kept stored with phenol, were tested to see if any would fall into the same class with that of rabbit 487. These are listed in the table as sera E to I, inclusive. The last two, H and I, were from guinea-pigs and the rest were from rabbits. Table 8, (C) shows the absorption from 100 units of sensitizer from each of these sera at the different temperatures. In table 8, (D), is shown the absorption from 200 units. Of these four sera, F and I proved to be in the same class as that of rabbit 487; i.e., about room temperature was the optimum for absorption.

Those investigators, therefore, who claim that either room temperature or 37° is the best for the sensitization of the cells, were doubtless right in their observations, but any generalization that all sera react in the same way is obviously incorrect. The best temperature for the sensitization of the cells seems, rather, to be a variable, its absolute value depending on the particular lot of serum used in the test. No explanation of this variability is here advanced.

V. RELATION OF THE GLOBULIN CONTENT OF THE IMMUNE SERUM TO THE ABSORPTION OF THE ANTIBODIES

It was noted in section I that the absorption of hemolytic antibodies by sheep cells varies with the sera of different animals of the same species and of different species. The question at once arises as to the cause of this variation.

Manwaring (16) has shown that serum proteins may be absorbed by the red cells of another species. It has long been known that the immune bodies are, in most cases, thrown out of the serum along with the globulin fractions, and Hurwitz and Meyer (17 and 18) have shown that these globulins are, as a rule, although not necessarily, increased in varying amounts during the process of immunization. The increase is, in their opinion, due to, and roughly proportional to the amount of metabolic disturbances set up in the animal. The evident relation of the globulins to the antibodies, in these respects, suggested a possible connection with the absorption of the antibodies by the cell-antigen. The experiments recorded in this section were undertaken with this idea in mind.

The sera of rabbits and guinea-pigs were used in this work, and quantitative determinations of the serum proteins were made on each lot of serum before the adsorption tests were done. The protein determinations were made according to the microrefractometric method of Robertson (19), with an Abbe refractometer. Serum, inactivated at 56°C., was always used immediately to avoid the possibility of bacterial contamination, and special attention was given to the cleaning of the glass-ware.

The results of this work are given in table 9.[2] There is no evidence, from these, that the amounts of the serum proteins present have any relation to the amount of sensitizer absorbed by the cells. Rabbits 481 and 486 were bled and tested at intervals for 116 days after the beginning of the immunization.

TABLE 9

Absorption from immune sera with quantitative determinations of the serum proteins

UNITS ABSORBED IN		CONCENTRATION OF ANTIBODY UNITS PER CC. IN THE SERUM DILUTIONS					TITER	GLOBULIN	ALBUMIN
Test	Animal	20	50	100	200	500			
								per cent	*per cent*
1	Rabbit 276		50	99	193		8,000	1.71	5.73
2	Rabbit 374		45	80	150		2,000	1.82	4.26
3	Rabbit 471		47	87	150		2,500	1.16	5.6
4	Rabbit 487		49	95	193		40,000	1.48	5.62
5	Rabbit 481			97	175		10,000	1.95	4.48
6	Rabbit 481		30	50	91	160	1,500	3.24	2.63
7	Rabbit 481	18	30	55	100		800	2.7	4.18
8	Rabbit 481	15	23	37			600	2.74	4.76
9	Rabbit 481	18	30	55	102		500	2.34	4.6
10	Rabbit 481	17	35	65	120		500	2.81	4.12
11	Rabbit 481	20	42	76	140		500	3.92	4.15
		Animal emaciated, died soon after							
12	Rabbit 486			94	180	410	8,000	2.41	4.01
13	Rabbit 486		30	52	91	160	1,500	3.47	2.73
14	Rabbit 486		40	80	150		1,000	2.2	4.5
15	Rabbit 486	19	38	71			800	1.96	5.38
16	Rabbit 486	18	30	55	102		500	2.19	4.8
17	Rabbit 486	17	35	65	120		600	2.77	3.85
18	Rabbit 486	18	40	71	134	370	600	3.42	4.25
		Animal emaciated, died soon after							
19	Guinea-pig	15	25	40	45		2,000	2.51	2.48
20	Guinea-pig	15	20	30	40		1,500	2.65	2.08
21	Guinea-pig		17	20	33		2,000	3.03	2.08

The globulins and albumins fluctuated considerably during that time, but the fluctuations seem not to bear any evident relation to

[2] The figures given in tables 9 to 12 do not all represent the actual experimental results. In many cases the concentrations tested did not coincide with those given in the tables, so the figures had to be interpolated from logarithmic charts, for the purpose of tabulation.

the absorption. At the time of the last bleeding both animals were emaciated and the globulins of both sera were very high. They were about the same in amount as at the second bleeding, but the absorption is much more complete.

VI. THE RELATION BETWEEN THE ABSORPTION OF THE ANTIBODIES AND THE TITER OF THE SERUM

It has been noted that the constants, K and n, vary in the absorption from the sera of different animals. It has also been observed, incidentally, that often the serum of the higher titer showed the higher absorption. In tests 2 and 3, of table 1, the serum was tested fresh and then tested again after four months' storage, during which time the titer dropped from 10,000 units to 6000 units per cubic centimeter. The later test gave a somewhat lower absorption than when the serum was first drawn. Another serum was tested with a titer of 40,000 units per cubic centimeter, and gave a very high absorption (test 9, table 1). The rabbit was kept and its serum drawn again eight days later (test 10, table 1). The titer had dropped to 25,000 units per cubic centimeter, and from all concentrations a marked lowering of the absorption was observed. It was thought, therefore, that there might be a relation between the titer of the serum and the number of antibody units the red cells would take up.

A number of fresh sera of both high and low titers were tested. Some of the animals having serum of high titer were tested subsequently, after the titer had dropped. The results are given in table 10. In many cases, the absorption seems to vary with the titer of the serum, but this is not constant, and the differences in the titers above 2000 units per cubic centimeter seem to have no definite relation to the absorption. Normal rabbit serum, in spite of its low titer, gives complete absorption from the undiluted serum (tests 6 and 8). Most of the other low-titered sera were obtained from rabbits that had formerly had high titers, and the absorption from these was uniformly low. Tests 17 to 21, however, were made with the sera of rabbits 72 and 80, drawn during the first few days of immunization. They all show uniformly

high absorption, although the titers vary from 100 to 16,000 units per cubic centimeter.

These results seem to indicate, therefore, that, although in many cases the higher the titer the higher the absorption, it is

TABLE 10

Relation of the absorption to the titer of the serum

UNITS ABSORBED IN		CONCENTRATION OF ANTIBODY UNITS PER CC. IN THE SERUM DILUTIONS						TITER
Test	Animal	10	20	50	100	200	500	
1	Guinea-pig		4	10	20			125
2	Guinea-pig	5	8		50			1,000
3	Guinea-pig	6	10	20	33			1,000
4	Guinea-pig	6	12	24	40			1,000
5	Guinea-pig	5	19	41	70	100		4,000
6	Rabbit	10	20	50				50
		Normal, untreated						
7	Rabbit	10	18	30	44	77		1,000
8	Rabbit	10	20	50	100			100
		Normal, untreated						
9	Rabbit			45	80	150		2,000
10	Rabbit		18	35	55	100		800
11	Rabbit	10	19	37	70	125		800
12	Rabbit	10	20	47	92	160	340	10,000
13	Rabbit			47	90	167	375	6,000
14	Rabbit	10	20	47	86	160	300	6,000
15	Rabbit			47	92	150	375	4,000
16	Rabbit		19	46	88	150	300	4,000
17	Rabbit 72			50	100			100
		Animal three days immune						
18	Rabbit 72				99	194	450	1,000
		Animal six days immune						
19	Rabbit 72					198	485	16,000
		Animal ten days immune						
20	Rabbit 80				91	175	350	800
		Animal five days immune						
21	Rabbit 80				96	188	426	8 000
		Aminal nine days immume						

not the titer of the serum, per se, which causes the variation. Rather, it would indicate that the extent of absorption is dependent on the length of time the animal is immune, the higher absorption being obtained early in the period of immunity.

This variation may be caused by some inhibiting substance, which is present in the serum in larger quantities later in the immunity period.

VII. SELECTIVE ABSORPTION

In the preceding section, it was indicated that the variation in absorption may be due to some disturbing element in the serum, the lowering of the absorption depending on the amount of this substance present. It was not possible to show any influence of the fluctuations of the serum proteins, so evidently the disturbance is not due to the serum proteins as such. The problem now is to determine the nature of this element, if possible, and its mode of action, i.e., whether it is taken up by the cell, or acts merely by virtue of its presence in the serum.

It has been noted before that, when the logarithmic plots of the absorption are made, some of the curves represent straight lines while others do not. Curve A, figure 3, for which $k = 165.9$, approaches the straight line very closely. Curve B, in the same figure, represents a test made on the serum of the same rabbit, drawn six days later. The titer had dropped from 20,000 units to 16,000 units per cubic centimeter with a drop in the absorption constants, $k = 80.73$. The plot, as is seen, deviates considerably from the straight line, the deviation being most pronounced in the higher concentrations. Figures 4 and 5 represent comparatively high values for K, but in neither is it so high as in curve A, figure 3. These plots, also, deviate from the straight line in the higher concentrations of antibody units. Figures 6 to 9 all represent very low values for K, and in these is seen a drop in the curve in the intermediate concentrations.

This deviation of the curve from the straight line, according to a physical interpretation, means that there is a selective absorption of the antibodies, the amount of selection differing with the concentration. Selective absorption may, although it does not necessarily, denote the presence of two or more substances in the serum capable of being absorbed.

It was tentatively assumed, therefore, that the variations in the absorption are caused by the presence of some inhibiting sub-

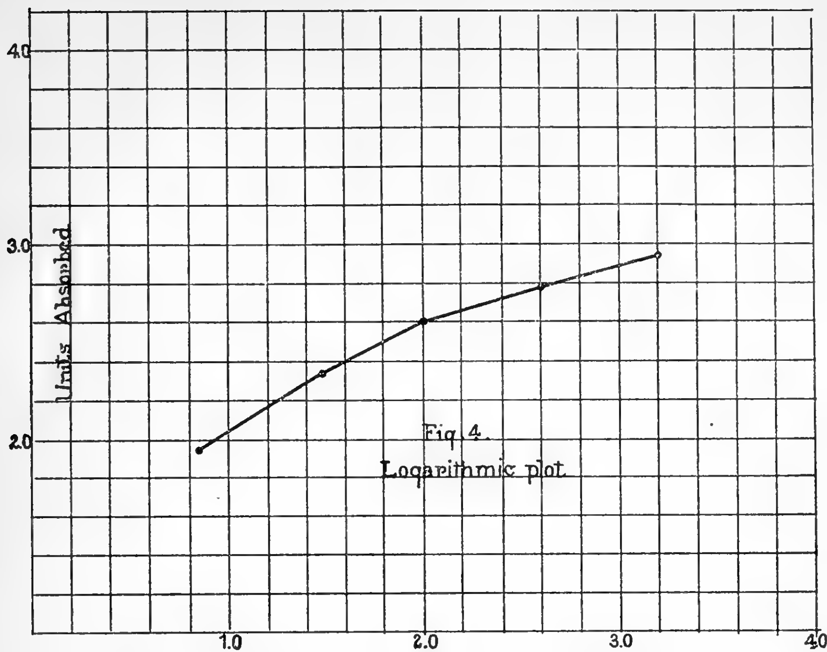

FIG. 4. LOGARITHMIC PLOT OF THE DATA OF TEST 5, TABLE 9
$K = 65.32,\ n = 0.37$

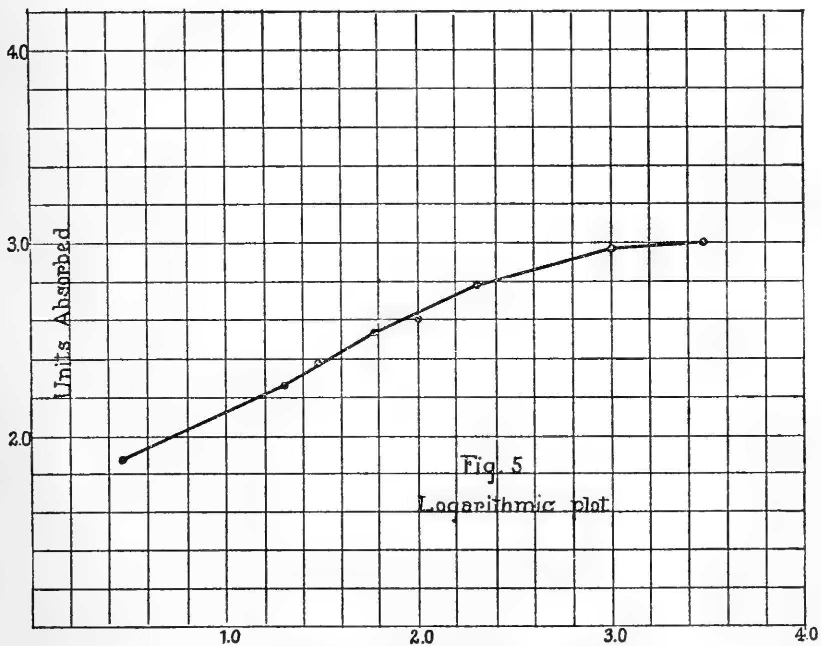

FIG. 5. SIMILAR PLOT OF TEST 12, TABLE 9
$K = 35.0, n = 0.51$

stance in the serum; and that this substance exerts its influence by virtue of its own absorption by the cell. The following "multiple absorption" experiments were carried out to support or disprove this hypothesis.

Sheep cells, one unit, were allowed to take up a certain number of units of sensitizer from a serum that, from previous tests, had been found to have a comparatively high absorption constant. (The value of K was not determined exactly in these experiments,

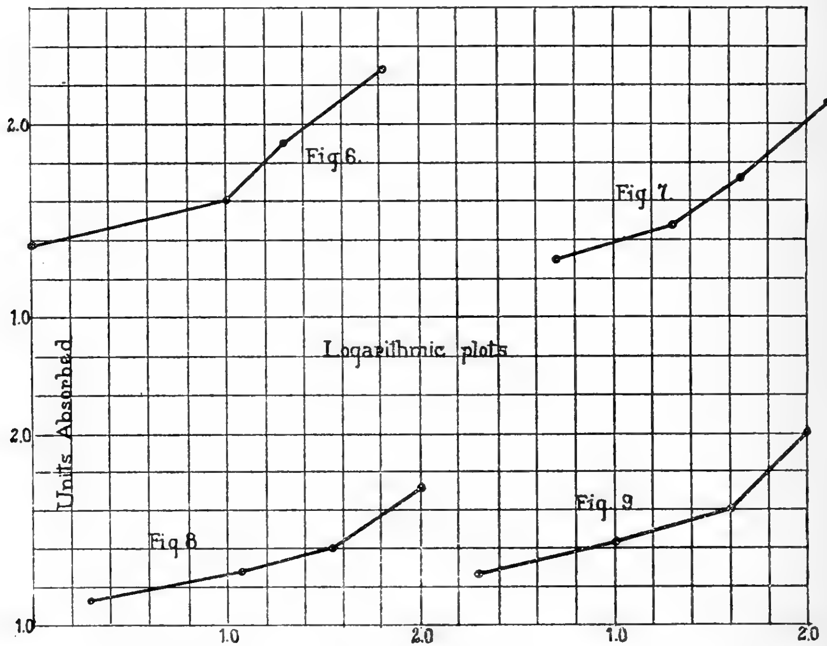

FIG. 6. LOGARITHMIC PLOT OF THE DATA OF TEST 14, TABLE 9
$K = 24.0, n = 0.58$

FIG. 7. SIMILAR PLOT OF TEST 16, TABLE 9
$K = 6.53, n = 0.56$

FIG. 8. SIMILAR PLOT OF TEST 8, TABLE 9
$K = 8.0, n = 0.31$

FIG. 9. SIMILAR PLOT OF TEST 7, TABLE 9
$K = 15.0, n = 0.41$

but only estimated to be high or low in proportion to the amount of absorption from the concentrations tested.) Another similar lot of cells was allowed to take up sensitizer from a serum having a lower absorption constant. Both lots of cells were then separated from the supernatant liquid by centrifuging at low speed, and each treated with an equal number of units of sensitizer from one serum. The amount of absorption was determined as in the preceding experiments.

If this inhibiting element is really taken up by the cells, those which had received a certain number of units of sensitizer from a serum with a low absorption constant should be more resistant to further sensitization than cells which had received an equal number of units from a serum with a higher absorption constant. The results of the experiments described below agree with the expectations.

Experiment 1

a. First absorption. Set 1:[3] One unit of sheep cells was treated with 50 units of fresh guinea-pig immune serum, titer 1/150. Titration of the supernatant liquid showed only 5 units absorbed. The value of K, therefore, is very low. Set 2: One unit of sheep cells, treated with 50 units of guinea-pig immune serum, titer 1/5000, absorbed 20 units of sensitizer. The value for K, is therefore, much higher than for the serum of set 1, although still rather low.

b. Second absorption. Each lot of cells was then treated with 50 units of the second serum, titer 1/5000. By titration of the supernatant liquid, lot 1 was found to have taken up 5 more units, while lot 2 absorbed 10 more. The serum with the lower absorption constant was therefore, more effective in preventing further sensitization of the cells than the serum with the higher absorption constant.

Experiment 2

a. First absorption. Set 1: One unit of cells, treated with 100 units sensitizer from rabbit serum, titer 1/1200, absorbed 50 units. The value for K is, therefore, comparatively low. Set 2: One unit of cells, treated with 100 units sensitizer from rabbit serum, titer 1/5000, took up 90 units. The value for K then is comparatively high.

[3] All sets were made in duplicate.

b. Second absorption. Each lot of cells, after separation from the supernatant liquid, was treated with 100 units sensitizer from the second serum, titer 1/5000. Lot 1 took up so small an amount in this second absorption that it was not titratable, while lot 2 took up 25 more units. Here, 50 units taken up from the serum with the lower absorption constant were more effective in preventing further senitization of the cells than 90 units from the second serum.

Experiment 3

a. First absorption. Set 1: One unit cells, treated with 100 units sensitizer from rabbit serum, titer 1/600, absorbed completely 100 units. The value of K is high then, in spite of the low titer. Set 2: One unit of cells, treated with 200 units sensitizer from rabbit serum, titer 1/4000, absorbed 100 units. The value for K, therefore, is much lower than for the serum of set 1.

b. Second absorption. Each lot of cells was then treated with 200 units of sensitizer from the second serum, titer 1/4000. Cells of lot 1 absorbed 75 more units, and those of lot 2 absorbed 30 more. The serum with the lower absorption constant is here, also, the more effective in making the cells resistant to further sensitization.

Experiment 4

a. First absorption. Set 1: One unit of cells, treated with 200 units of sensitizer from rabbit serum, titer 1/4000, absorbed 80 units. The absorption constant is very low. Set 2: One unit of cells, treated with 120 units sensitizer from rabbit serum, titer 1/5000, took up 120. The absorption constant, therefore, must be very high.

b. Second absorption. Each lot of cells, after separation from the supernatant liquid, were treated with 120 units from the second rabbit serum, titer 1/500. Lot 1 absorbed 40 units more, while lot 2 took up 110 more. In this case, also, the absorption from the serum having the lower value for K[4] is much more effective in making the cells resistant to further sensitization than absorption from the serum with the high value for K.

In every case, in the foregoing experiments, the antibodies taken up by the cells from the serum with the lower absorption

[4] Where the plot deviates from the straight line the values of K and n can be determined only approximately.

constant had much more influence in inhibiting further sensitization of the cells than a larger number of antibodies taken up from the serum with the higher absorption constant. It is obvious, therefore, that the inhibiting element, whatever its nature, can be taken up by the cells, and probably in proportion to its combining affinity, inhibits the absorption of the antibodies.

VIII. VARIATION OF THE ABSORPTION WITH THE LENGTH OF THE PERIOD OF IMMUNITY

In the foregoing section, it was found that the disturbing element, which inhibits the absorption of the antibodies, is actually taken up by the cells along with the antibodies. In section VI, it was indicated that the amount of this substance probably varied during the time that the animal is immune. In this section, it is purposed to show how the absorption varies during the immunity of the animal, which variation seems to give some indication as to the nature of the disturbing factor.

A careful record of rabbits 481 and 486 was kept while testing for the relation of the serum proteins to absorption. The animals were given three injections within the first nine days and no more during the 116 days they were under observation. Table 11 shows the fluctuations in the absorption from their sera, also the titers and the number of days after the first injection when the serum was drawn. In figures 10 and 11 are plotted the observations.

The first tests, made on the sixteenth day, showed a high titer and a comparatively high absorption. The second test, on the twenty-fourth day, showed a great drop in the titer and a corresponding drop in the absorption. Following the initial drop, the titer dropped very gradually until about the sixty-second day, and from then on held fairly constant until the last observation on the one-hundred and sixteenth day. Further observations were not possible on account of the death of the animals. In the case of rabbit 481, where the titer dropped gradually, or held almost constant, the absorption, although fluctuating somewhat, also held fairly constant, and toward the end of the period

of observation, showed a distinct rise. The fluctuations of the absorption from the serum of rabbit 486 were more pronounced, showing a marked rise after the initial drop, with a not so marked rise at the end. It is highly probable, however, judging from other observations described below, that the rise at the end

TABLE 11

*Absorption tests on the sera of rabbits 481 and 486, drawn at intervals during a period of immunity of 116 days**

	CONCENTRATION OF ANTIBODY UNITS PER CC. IN THE SERUM DILUTIONS					TITER	DAYS IMMUNE
	50	100	200	500	1000		
Units absorbed from serum of rabbit 481:							
Test 1		94	175	400	600	10,000	16
Test 2	30	50	91	160		1,500	24
Test 3	30	55	100			800	62
Test 4	23	37	81			600	80
Test 5	30	45	98			500	91
Test 6	30	50	110			500	110
Test 7	42	76	140	330		500	116
Units absorbed from serum of rabbit 486:							
Test 1		94	180	410	670	8,000	16
Test 2	30	52	91	160		1,500	24
Test 3	40	80	150			1,000	62
Test 4	38	71				800	80
Test 5	30	55	102			500	91
Test 6	35	65	120			600	110
Test 7	40	71	135	370		600	116

* These rabbits received three injections of washed sheep corpuscles within the first nine days. Each injection consisted of 3 cc. of the cells, measured in terms of whole blood.

would have continued higher, had it been possible to make further tests.

The results from these two rabbits seemed significant, so a number of others were started. Unfortunately, all except one, rabbit 69, died so their history is not recorded here. A goat was also immunized and its history followed. Table 12 and figures 12 and 13 show the observations on these animals.

The results agree very well with those obtained with the other two rabbits. Even though the titer is not high at first, the absorption is high, with a great drop within the second or third week of the period of immunity. Following this, the absorption

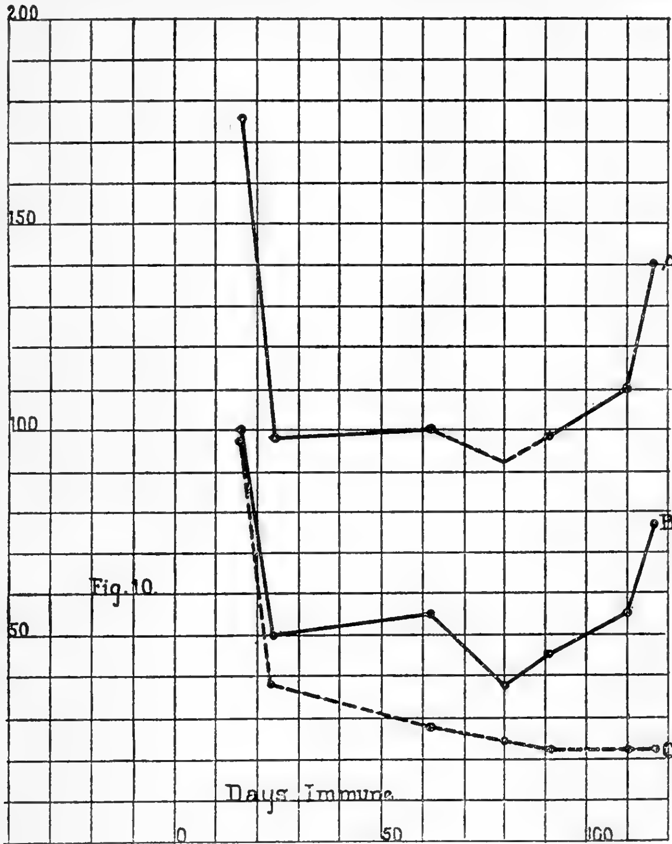

Fig. 10. Plots of the Absorption from the Serum of Rabbit 481 Against the Time of Immunity. See Table 11

Curve A represents the absorption from 200 units, B, from 100 units, and curve C represents the square root of the titer.

holds about constant for a time, after which it shows a more or less gradual rise.

The history of these animals seems highly significant in suggesting an explanation as to the nature of the disturbing factor in

the absorption. In nearly every case, the sudden initial drop in the absorption is accompanied by a similar drop in titer. In the case of the goat, this is not evident, but seven days had elapsed

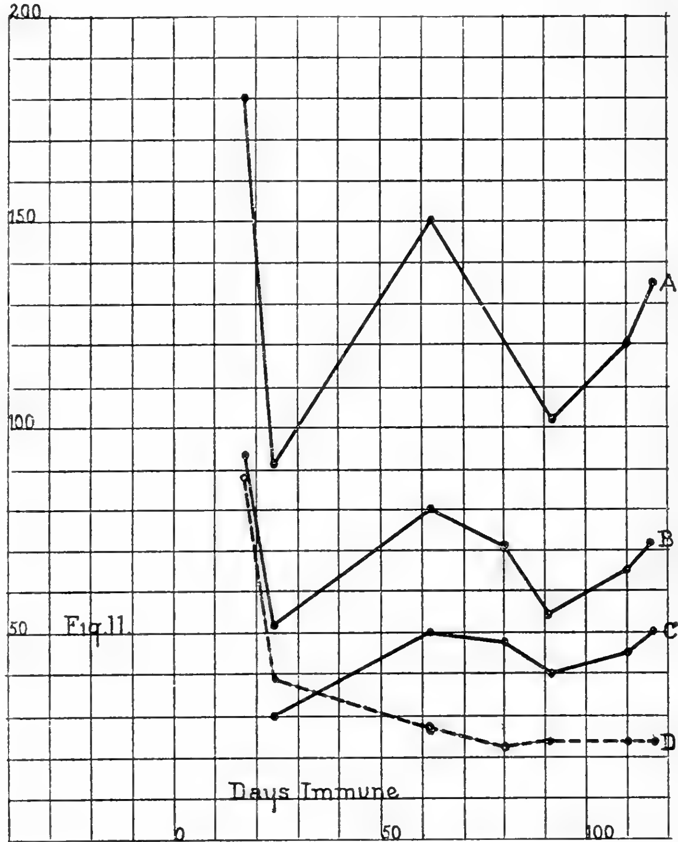

Fig. 11. Similar Plot for Rabbit 486, Table 11

Curve C represents the absorption from 50 units, and D represents the square root of the titer.

between the first and second tests, during which the titer may have gone higher and dropped back to 1/2000.

It is conceivable that, as the amboceptor content of the animal's blood-stream drops, the destruction of the antibody occurs in progressive stages, the first stage being merely the loss of the

power to sensitize the cells to the action of complement. The ability to combine with the cell would still be retained for a time. This would be, in effect, somewhat analogous to the "agglutinoids" of Ehrlich, which were worked out so thoroughly by Eisenberg and Volk (22). In titrating the serum, then, these deterioration products of the amboceptor would not be detected, and one hemolyzing unit would contain, in addition to the antibodies, a variable amount of the deterioration products. Those which still retained their affinity for the antigen would, in the absorption experiments, tend to cause a lowering of the absorption of

TABLE 12

Absorption tests on the sera of rabbit 69 and a goat, drawn at intervals during the period of immunity

	CONCENTRATION OF ANTIBODY UNITS PER CC. IN THE SERUM DILUTIONS					TITER	DAYS IMMUNE
	50	100	200	500	1000		
Units absorbed from serum of rabbit 69:*							
Test 1		99	194	450		1,000	6
Test 2			198	485	875	20,000	10
Test 3			194	452	690	16,000	16
Test 4		60	100	200	300	2,000	27
Test 5		75	120	250	400	1,000	37
Test 6		75	125	250		800	45
Test 7		75	130	275	400	600	62
Test 8		86	150	350	500	600	86
Units absorbed from serum of goat:†							
Test 1	49	96	180			1,000	10
Test 2	20	30	70			2,000	19
Test 3	40	70	100			1,000	49
Test 4	40	70				1,000	63
Test 5	40	70	100			1,000	65
Test 6	45	75	120			1,000	92
Test 7	47	85	150			800	120

* Rabbit 69 received two injections with a six-day interval.

† The goat received three large injections of about 8 cc. each and ten very small injections of about 1.5 cc. each. The last injection was one month before the final test.

the antibodies. The fact that the greatest lowering of the absorption is coincident with the most rapid destruction of the antibodies is very forcibly suggestive that the substance in the serum

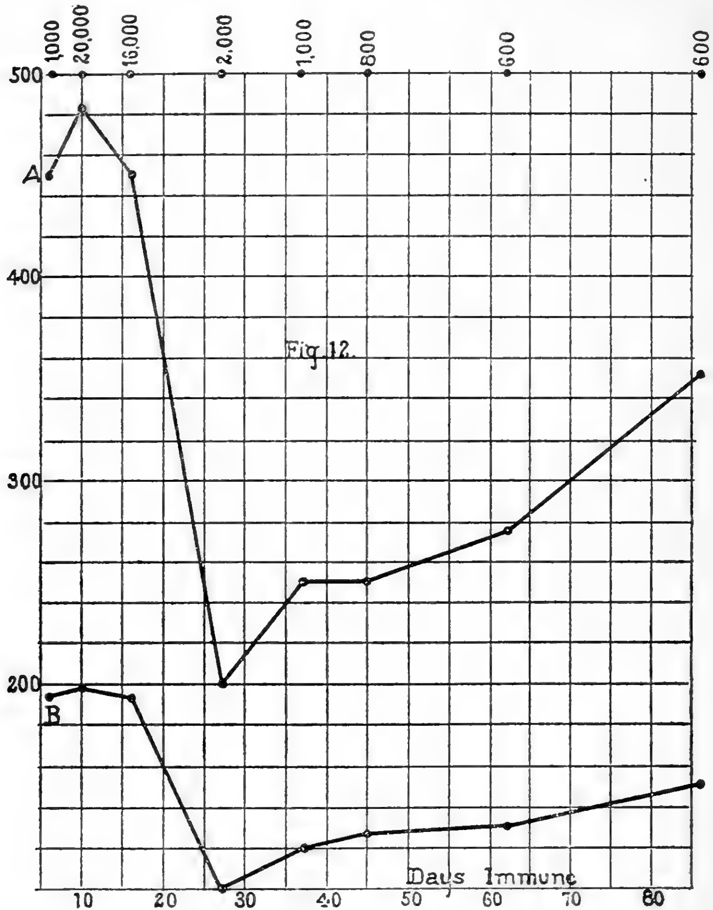

FIG. 12. SIMILAR PLOT FOR RABBIT 69, TABLE 12

Curve A represents the absorption from 500 units, and B from 200 units. The titers of the sera on the various days is indicated at the top of the chart.

which causes this disturbance may be defined as the product of the destruction of the antibodies.

IX. DISCUSSION

It has been found that, in the absorption of hemolytic immune bodies by red cells, immense quantities, as much as 2000 units or more, may be taken up by one unit of cells, provided their con-

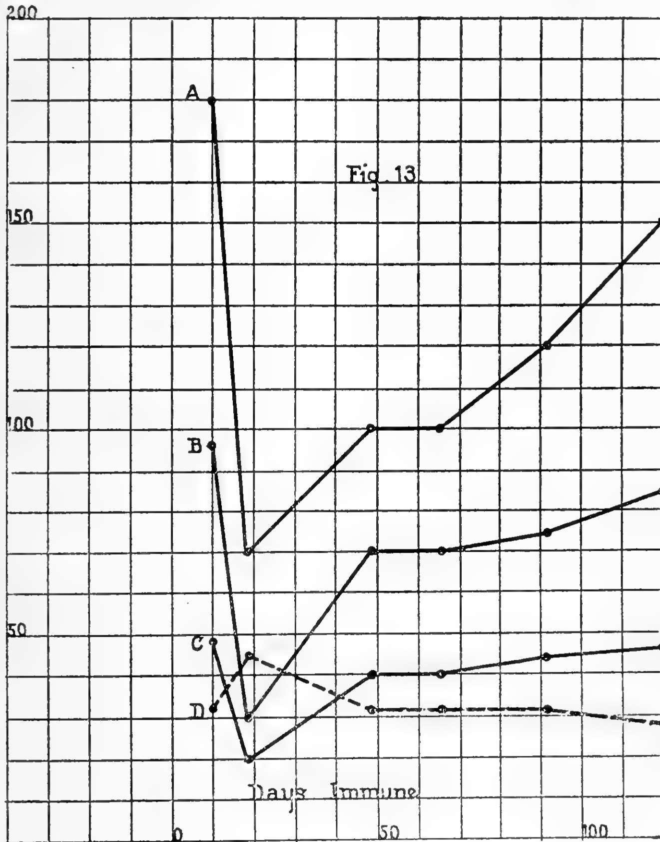

Fig. 13. Plot Similar to Figure 11, for the Goat

See table 12

centration in the surrounding medium is sufficiently high. On the other hand, all the antibodies are often not taken up even from concentrations as low as 50 units per cubic centimeter. This is certainly not in accordance with the theory of chemical valencies. It has been found, moreover, that this absorption follows,

more or less strictly, the physical law proposed by Arrhenius, which applies to the distribution of a solute between two immiscible solvents. The equation, $B = KC^n$, which represents this law, could, however, with equal accuracy, be applied to simple adsorption phenomena, such as the taking up of acetic acid from a benzol solution by silica gel. Arrhenius assumed that all absorption tests with the hemolytic system would follow this law within the limits of experimental error, but it was shown by Manwaring, and confirmed in this paper, that many, in fact, most of the absorption tests do not follow this law strictly, as is shown by the deviation of the logarithmic curves from the straight line.

One of the most striking things about the absorption of hemolytic antibodies is the great differences obtained with the different sera. While with many sera the absorption is very low, incomplete even from a concentration of antibodies as low as 20 units per cubic centimeter, with other sera the absorption is practically complete from as much as 200 units per cubic centimeter.

The antigen-antibody combination takes place with great rapidity. A large proportion of the antibodies are absorbed almost instantly. Equilibrium is practically established within five minutes, and in every case within fifteen minutes whatever the concentration of the antibodies.

The variability of the absorption from the different sera is shown also in the extraction at the various temperatures. The extraction is always less at 0° than at room temperature, and becomes progressively less again as the temperature is raised to 45° and above. The differences in the different sera become evident in the absorption at room temperature and at 37°. Of the sera that were tested, the majority gave the highest extraction at 37° to 40°. Others showed the highest extraction at about room temperature, 15° to 25°. This indicates that the optimum temperature for the antigen-antibody combination is a variable, the absolute value of which depends on the particular lot of serum used in the test.

An endeavor was made to determine the cause of the extreme variations obtained in the absorption from the different lots of

serum. A large number of tests were made on the different sera, at the same time making quantitative determinations of the globulins and albumins. The quantitative fluctuations of the serum proteins were not shown to bear any direct relation to the variations in the absorption. They may, however, exert some more indirect influence which was not detected by this method.

Although in many cases, the serum with the lower titer gave the lower absorption, this was not constant, and with titers of 2000 units per cubic centimeter or higher the differences did not seem to bear any relation to the amount of sensitizer taken up by the cells. Normal sera and sera that were drawn early in the period of immunity gave uniformly high absorption, regardless of the titer. This indicates that the lower absorption comes about as a result of some change in the serum after the animal is immunized.

This lowered absorption is evidently due to some substance in the serum which is absorbed by the cells along with the sensitizer; it is probable, also, that, in proportion to the combining affinity of this substance, the absorption of the antibodies is inhibited. This is shown by the fact that cells, which take up sensitizer from a serum that gives a low absorption, are more resistant to further sensitization than an equivalent lot of cells sensitized with a larger number of antibody units from a serum that gives a high absorption. The serum, therefore, that gives the higher absorption, has the smaller percentage of this inhibiting substance, as compared to the number of antibodies.

It is true that the number of animals is not large, for which the history was kept of the absorption at intervals during the period of immunity. The results, however, are consistent. The fact that, in every case, the absorption is highest early in the immunity period, and that the greatest drop in the absorption is coincident with the most rapid drop in titer, is forcibly suggestive that the inhibiting substance represents the deterioration products of the amboceptor, i.e., the amboceptors which have lost their sensitizing power, but still retain their combining affinity for the antigen. This would be, in effect, somewhat analogous to the "agglutinoids" of Ehrlich. This hypothesis would account, not only, for

the great differences in the absorption from the different sera, but also for the fact that so many of the tests fail to conform to the equation proposed by Arrhenius.

X. SUMMARY

1. Red cells, when treated with their specific immune serum, take up amounts of sensitizer which vary with the concentration of the sensitizer in the surrounding medium. Although from very low concentrations, all is not absorbed, as much as 2000 units or more may be taken up by one unit of cells, provided the concentration of the antibodies is sufficiently high.

The logarithmic plots of some of the tests approach a straight line very closely, while many deviate considerably from the straight line. The amount of deviation of the curve is proportional to the amount of deviation of the tests from the physical law proposed by Arrhenius, which is expressed by the equation, $B = KC^n$. The amount of absorption varies also with the serum used. Some given practically complete extraction from as much as 200 units per cubic centimeter while with other sera, all is not absorbed from concentrations of antibodies as low as 50 units per cubic centimeter.

2. The combination of the red cell and its antibody is extremely rapid. In every case, fifteen minutes were found to be sufficient time for equilibrium to be established.

3. With the majority of sera the extraction of sensitizer by the cells was found to be greatest at a temperature of about 37°C. Some sera, however, gave more complete absorption at 15° to 25°C. than at any other temperature.

4. Neither the quantitative fluctuations of the serum proteins nor the titer of the serum was found to have any constant relation to the variations in the absorption.

5. Cells, sensitized with a serum which gave a low absorption, were more resistant to further sensitization than cells, sensitized with a much larger number of units from a serum which gave a high absorption. This indicates that the substance in the serum, that inhibits the absorption, is really absorbed by the cell along with the antibodies.

6. In every case tested, the extraction of the antibodies was greatest from serum drawn early in the period of immunity, regardless of the titer. The greatest drop in the absorption was found to be coincident with the most rapid drop in the titer.

7. The accumulated evidence is forcibly suggestive that the great variations in absorption are due to the presence of the deterioration products of the antibodies, i.e., the antibodies which have lost their sensitizing power but still retain their affinity for the antigen.

The writer's thanks are due Dr. R. R. Hyde, under whose direction the work has been carried forward.

REFERENCES

(1) Ehrlich, Paul: Studies in Immunity. 1910. Translation by Charles Bolduan.

(2) Arrhenius, S.: Immunochemistry. MacMillan, 1907, 144–166; 257–259.

(3) Bordet, J.: Studies in Immunity. Translation by Frederick P. Gay. First Ed., 1909, 440.

(4) Manwaring, W. H.: The absorption of hemolytic amboceptor. Jour. Inf. Dis., 1905, **2**, 485.

(5) Manwaring, W. H.: The third serum component. Jour. Inf. Dis., 1906, **3**, 647.

(6) Manwaring, W. H.: Analytical methods of serum pathology. Jour. Biol. Chem., 1905–1906, **1**, 213.

(7) Manwaring, W. H.: On the so-called physical chemistry of the hemolytic serum. Jour. Inf. Dis., 1907, **4**, 219.

(8) Manwaring, W. H.: Qualitative changes in the third serum component. Jour. Inf. Dis., 1908, **5**, 55.

(9) Coulter, C. B.: The isoelectric point of red blood cells and its relation to agglutination. Jour. Gen. Physiol., 1920–1921, **3**, 309.

(10) Coulter, C. B.: The equilibrium between hemolytic sensitizer and red blood cells in relation to the H-ion concentration. *Ibid.*, p. 513.

(11) Kahn, R. L., and Lyon, D. S.: Studies on complement fixation. IV. On the affinity of sheep corpuscles for anti-sheep hemolysin. Jour. Inf. Dis., 1921, **29**, 651.

(12) Bull, C. G.: The agglutination of bacteria in vivo. Jour. Exp. Med., 1915, **22**, 484.

(13) Neil, M. H.: The complement fixation test for syphilis. Pub. Health Reports, 1918, **33**, 1387.

(14) Hinton, Wm. A.: A standardized method of performing the Wassermann reaction. Am. Jour. Syph., 1920, **4**, 598.

(15) Kolmer, J. A., Rule, A. M. and Trist, M. E.: Studies in the standardization of the Wassermann reaction. XII. *Ibid.*, p. 616.

(16) Manwaring, W. H., and Yoshio Kasuma: Protein absorption by blood corpuscles. Proc. Soc. Exp. Biol. and Med., 1916, **13**, 173.

(17) Hurwitz, S. H., and Meyer, K. F.: Studies on the blood proteins. Jour. Exp. Med., 1916, **24**, 516.

(18) Hurwitz, S. H., Meyer, K. F. and Taussis, L.: Studies on the blood proteins. III. Jour. Inf. Dis., 1918, **22**, 1.

(19) Robertson, T. B.: A microrefractometric method of determining the percentages of globulin and albumin in very small quantities of blood serum, Jour. Biol. Chem. 1915, **22**, 235

(20) Huntoon, F. M. et q. : Antibody studies. I, II, and III. Jour. Immun., 1921, **6**, 117–200.

(21) Rokuro Umemura: A serological study of cholera immunity. Jour. Immun., 1920, **5**, 465.

(22) Eisenberg, P. and Volk, R.: Zeitschr. f. Hyg., 1902, **40**, 155.

(23) Amato, A. Ricerche Sperimentali sulla fagocitosi, Sperimentale, Florence, 1917, **71**, 459.

STUDIES ON THE TOXICITY OF HUMAN BLOOD PLASMA FOR GUINEA-PIGS

I. RELATIVE TOXICITY OF FETAL AND MATERNAL PLASMA

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In a recent paper Sachs and Oettingen (1) call attention to certain striking differences in maternal as contrasted with fetal plasma. Their observations disclosed that the surface tension, as well as the relative stability of the plasma colloids, as determined by coagulation with heat, salts, alcohol, etc., was distinctly altered. They emphasize the fact that in our usual serological studies we ignore the rôle of the largest and most unstable colloidal aggregate—fibrinogen—and that it is only when we work with plasma that we more closely approximate normal biological conditions. Particularly in relation to the Wassermann reaction their findings are of significance when we recall that the reaction is not infrequently nonspecifically positive in the pregnant woman, while fetal serum on the contrary, is frequently negative despite positive clinical evidence of syphilis. This they believe may be related to the fact that the colloids of the pregnant blood flocculate more readily than do those of the fetal blood, therefore favoring nonspecific colloidal alterations.

In view of the definite alterations that Sachs and Oettingen have found we thought it would be of interest to determine whether fetal and maternal plasma differed in toxicity when injected intravenously into guinea pigs. Numerous studies have been reported dealing with primary serum toxicity when

the serum of one species is injected into another; so, too, a number have been reported that concern the relative toxicity of human serum (normal and from pathological cases) for the guinea-pig, but we have found no reference in the literature dealing with the particular phase of the question that we have under consideration.

Insofar as we inject a colloidal mixture we must be prepared to meet with the usual mode of death that follows injections (in sufficient dosage) of any number of the more typical colloidal substances usually used in experimental work. Hanzlik and Karsner (2) have published an extensive study of the effects of the intravenous injection of various colloids which produce an "anaphylactoid" death, while Friedberger (3), Nathan (4), Novy (5), and others have studied in detail the phenomena associated with the injection of kaolin, starch, agar, etc. Related to the phenomenon that we have under consideration must be considered the toxicity manifested by organ extracts when intravenously injected; the literature of this particular field is an extensive one.

METHODS

The maternal blood was collected shortly before delivery, the blood being obtained from the median basillic vein. The fetal blood was obtained from the umbilical cord.

The blood was allowed to flow to the 10 cc. mark in a graduated centrifuge tube containing 2 cc. of a 2 per cent sodium citrate solution. The blood was then centrifuged and the plasma pippeted off into a perfectly clean tube and then immediately used. We are of the impression that the results are more accurate if the fresh plasma is used, for if the plasma is allowed to remain at room temperature or is kept in a very warm place for any length of time, colloidal changes occuring in the plasma may affect the ultimate outcome of the experiment. If a tendency of the blood plasma to clot was observed, the plasma was not used. Because of the relatively large amount of plasma needed for the experiments we collected at least two tubes of plasma whenever possible.

Surface tension. In determining the surface tension of the plasma we used the Traube Stalagmometer (static method). Table 1 shows the relationship of the surface tension of fetal, pregnant, and non-pregnant female plasma.

Toxicity. We determined the toxicity of the plasma by slowly injecting directly into the jugular vein of guinea-pigs in the usual

TABLE 1

Surface tension of blood plasma in maternal, fetal, and normal cases

CASE NUMBER	MATERNAL	FETAL	NORMAL FEMALE ADULTS	NORMAL MALE ADULTS
1	63	59	61	57
2	62	56	62	58
3	60	55	61.5	60
4	63.5	60	62	59
5	61	57	63	58
6	62	58.5		59.5
7	63	55		
8	62.5	59		
9	63.5	58		
10	60			
Average.....	62.05	57.5	61.9	58.5

TABLE 1A

Surface tension of blood plasma during various periods of pregnancy

CASE NUMBER	DURATION OF PREGNANCY	RESULT
	months	
1	3	61
2	7	61
3	8	63
4	8	63.5
Average.............		62.12

TABLE 1B

Surface tension of blood plasma before and after menstruation

BEFORE MENSTRUATION		AFTER MENSTRUATION	
Case number	Result	Case number	Result
1	60	1	59
2	63	2	62

manner, beginning usually with 1 cc. of plasma per 100 grams of guinea pig weight. If immediate death resulted the dose was decreased to 0.75 cc. of plasma per 100 grams of guinea-pig, and so on. If the original dose did not kill almost immediately (one to five minutes) the dose

was then increased. Tables 2 to 7 give the amount of plasma used in the various conditions to produce immediate death in the guinea-pig.

The shock resulting in these animals is not to be compared with a true anaphylactic shock, as these animals were not sensitized; it is a coagulation phenomenon that in many cases resembles an anaphylactoid shock. The phenomenon of coagulation toxicity will be described in a following paper.

RESULTS

The results obtained in these experiments, demonstrate the relative increase in toxicity of the maternal pregnant plasma, as compared with fetal plasma. The following is a detailed study of our investigation.

1. Toxicity of normal human plasma immediately before delivery. The blood was obtained approximately one-half to one hour before the expected time for delivery. Our results show (table 2) that plasma obtained in these conditions is very toxic to the guinea pig, in a dose even as low as 0.5 cc. per 100 grams of guinea pig weight. It will also be observed that the surface tension of this plasma is high.

When the heart and lungs are examined from those pigs that died immediately after the plasma was injected intravenously, a clot was found in the heart ventricles, and the lungs were markedly distended and engorged. On palpation the lungs feel like a resilient mass of tissue. In the animals dying later than 10 minutes after the plasma was injected intravenously, the heart did not contain a demonstrable blood clot, but the lungs were distended and the surfaces were frequently covered with petechial hemorrhages. As will be described in a subsequent paper, the mechanism of shock death resulting when such plasma is injected intravenously into a guinea-pig, is not due to a clumping of a foreign substance in the capillaries of the bronchioles, but results from a coagulation of the plasma within the pulmonary capillaries and smaller blood vessels. While blood plasma of pregnant women may contain an increased amount of fibrinogen, it is by no means to be concluded that this injected fibrinogen itself is the cause of the intravascular

TABLE 2

Toxicity of human plasma from normal pregnant cases immediately before delivery

CASE NUMBER	WEIGHT OF GUINEA-PIG	DOSE OF PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT	TOTAL DOSE OF PLASMA INJECTED	RESULT
	grams	*cc.*	*cc.*	
1	340	0.5	1.7	Died in 24 hours
	260	0.6	1.56	Died in 10 minutes
	280	0.7	1.96	Died in 3 minutes
2	285	0.5	1.7	Died in 24 hours
	280	0.7	1.96	Died in 5 minutes
	240	1.0	2.4	Immediate death
3	260	0.3	0.78	Alive at end of 24 hours
	260	0.5	1.3	Died in 10 minutes
	310	0.65	2.1	Immediate death
4	205	0.75	1.53	Died in 15 minutes
	335	1.0	3.35	Immediate death
5	300	0.5	1.5	Died in 24 hours
	300	0.75	2.25	Immediate death
	280	1.0	2.8	Immediate death
6	270	0.5	1.35	Died in 3 hours
	395	0.75	2.96	Immediate death
7	285	0.6	1.7	Died in 3 minutes
	280	0.7	1.96	Died in 3 minutes
	240	1.0	2.4	Died in 2 minutes
8	340	0.5	1.9	Died in 12 hours
	260	0.6	1.56	Died in 10 minutes
	280	0.7	1.96	Died in 3 minutes
9	280	0.5	1.4	Died in 7 hours
	260	0.75	1.95	Died in 3 minutes
	270	1.0	2.7	Immediate death
10	310	0.5	1.5	Died in 6 hours
	285	0.75	2.3	Died in 2 minutes
	250	1.0	2.5	Immediate death

coagulation. Welker (6) has shown that serum (rattle snake) from good clotting blood produced no fatalities in the guinea-pig with doses running up to 1 cc. injected intraperitoneally.

2. Toxicity of normal human fetal plasma. Fetal plasma used in these experiments was obtained from the blood of the umbilical cord coming from the placenta. In comparing our results, we used only the fetal and maternal plasmas obtained from the same case. It will be observed from the table of comparative toxicities that the maternal plasma is approximately 3 times as toxic as the fetal plasma. While 0.5 cc. of maternal plasma

TABLE 3

Toxicity of normal human fetal plasma

CASE NUMBER	WEIGHT OF GUINEA-PIG	DOSE OF PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT	TOTAL DOSE OF PLASMA INJECTED	RESULT
	grams	*cc.*	*cc.*	
1	330	0.7	2.3	Alive at end of 24 hours
	165	1.2	1.98	Alive at end of 24 hours
2	270	1.0	2.7	Alive at end of 24 hours
	210	1.2	2.52	Alive at end of 24 hours
3	250	1.5	3.75	Alive at end of 24 hours
4	290	1.0	2.9	Alive at end of 24 hours
	300	1.2	3.6	Alive at end of 24 hours
5	225	1.0	2.25	Alive at end of 24 hours
	265	1.5	3.97	Alive at end of 24 hours
6	270	1.0	2.7	Alive at end of 24 hours
	210	1.2	2.52	Alive at end of 24 hours
7	330	1.0	3.3	Alive at end of 24 hours
	165	1.2	1.98	Alive at end of 24 hours
8	280	1.0	2.8	Alive at end of 24 hours
	300	1.5	4.5	Alive at end of 24 hours
9	240	1.0	2.4	Alive at end of 24 hours
	290	1.5	4.35	Alive at end of 24 hours

per 100 grams of guinea pig kills almost immediately, 1.5 cc. of fetal plasma per 100 grams of guinea-pig does not kill the animal (table 3). With greater doses death may occur with autopsy findings similar to those observed with the maternal plasma.

3. Toxicity of normal human plasma during various stages of pregnancy. The blood used for investigation in this experiment was obtained from women in various stages of pregnancy, varying anywhere from three months to almost term. Table 4 shows our results, and it will be observed that blood plasma from

TABLE 4

Toxicity of human blood plasma during pregnancy (3 to 8 months)

CASE NMMBER	DURATION OF PREGNANCY	WEIGHT OF GUINEA-PIG	DOSE OF PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT	TOTAL DOSE OF PLASMA INJECTED	RESULT
	months	*grams*	*cc.*	*cc.*	
1	7	320	0.5	1.6	Died within 24 hours
		200	0.75	1.5	Immediate death
2	8.5	340	0.5	1.7	Died within 24 hours
		235	0.75	1.76	Immediate death
		340	1.0	3.4	Immediate death
3	8	270	0.75	2.02	Immediate death
		245	0.5	1.22	Died within 24 hours
4	3	210	1.0	2.10	Immediate death
		245	0.75	1.83	Died within 24 hours

pregnant women during the earlier months of pregnancy is as toxic as blood plasma from pregnant women at time during delivery. Just how soon the blood in pregnancy becomes toxic we have not been able to ascertain. In one case of the third month of pregnancy the toxicity of the blood was not very great.

4. Toxicity of normal human male and female plasma. For comparative purposes it was of interest to observe the relationship of the degree of toxicity that normal human male and female plasma may have with that of fetal and pregnant plasmas. For this purpose normal healthy medical students were used. The surface tension of this plasma was intermediate between fetal

and maternal plasmas. The toxicity of the normal plasma was less than that of the maternal plasma (table 5).

The condition of the lungs in the guinea-pigs dying from the toxic dose of normal human plasma is the same as that found in the case of pregnant plasma. Inasmuch as the mode of death

TABLE 5

Toxicity of human plasma from normal cases

CASE NUMBER	WEIGHT OF GUINEA-PIG	DOSE OF PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT	TOTAL DOSE OF PLASMA INJECTED	RESULT
	grams	*cc.*	*cc.*	
1	295	0.5	1.47	Alive at end of 24 hours
	315	0.7	2.1	Alive at end of 24 hours
	220	1.0	2.2	Died in 24 hours
	210	1.2	2.25	Died in 24 hours
	350	1.5	5.25	Immediate death
2	105	0.75	0.78	Died in 24 hours
	125	1.0	1.2	Died in 24 hours
	105	1.5	1.5	Immediate death
3	445	0.75	3.33	Died in 24 hours
	365	1.0	3.65	Died in 1 hour
	185	1.5	2.77	Immediate death
4	105	0.75	0.78	Died in 24 hours
	150	1.5	2.25	Immediate death
5	290	1.5	4.35	Immediate death
	275	1.2	3.3	Immediate death
	190	1.0	1.9	Alive at end of 24 hours
6	250	1.5	3.75	Alive at end of 24 hours
	205	1.75	3.58	Immediate death

in the guinea pig injected with maternal and normal human plasmas is the same, the differentiation being one of degree, the toxic factor in the maternal plasma which causes the immediate death of the guinea pig must be present in the fetal and non-pregnant human bloods as well.

5. Toxicity of normal human female plasma before and after menstruation. Table 6 shows that the blood plasma before

TABLE 6

Toxicity of normal human (female) plasma before and after menstruation

CASE NUMBER	WEIGHT OF GUINEA-PIG	DOSE OF PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT	TOTAL DOSE OF PLASMA INJECTED	RESULT
a. Before menstruation				
	grams	*cc.*	*cc.*	
1	195	1.2	2.34	Died in 1 minute
	235	1.0	2.35	Died in 43 minutes
2	240	1.0	2.4	Immediate death
	235	0.75	1.76	Immediate death
	225	0.5	0.62	Alive at end of 24 hours
3	270	1.0	2.7	Immediate death
	210	0.75	1.57	Died in 2 hours
4	220	1.0	2.2	Immediate death
	230	0.75	1.72	Immediate death
	205	0.5	1.02	Died in 24 hours
5	210	0.75	1.577	Alive at end of 24 hours
	240	1.0	2.4	Alive at end of 24 hours
	200	1.25	2.5	Died in 3 hours
	160	1.5	2.4	Immediate death
b. After menstruation				
1	225	1.0	2.25	Immediate death
	190	0.75	1.42	Immediate death
	225	0.5	1.12	Died in 24 hours
2	340	0.75	2.25	Immediate death
	260	0.5	1.3	Alive at end of 24 hours
3	250	1.0	2.5	Died in 12 hours
	265	1.25	3.31	Died in 12 hours
	260	1.5	3.9	Immediate death
4	260	0.75	1.95	Immediate death
	230	0.5	1.15	Died in 12 hours
5	225	1.0	2.25	Died in 12 hours
	210	1.25	2.62	Immediate death

menstruation is a little less toxic than normal female plasma, while blood plasma following menstruation is almost as toxic as pregnant blood plasma.

Jacobi in 1876 advanced the theory that the metabolic processes in women present a distinct rhythm, gradually increasing in intensity up to the time of the menstrual flow, when they suddenly drop and reach their lowest point; after this they gradually rise again to attain their maximum intensity before the next menstrual period (Webster (7), Ott (8), Van der Velde (9)). These changes include in the blood—settling of blood

TABLE 7

Table of average toxicities of human blood plasma in pregnancy, fetal, and normal cases

CONDITION		AVERAGE TOXIC DOSE
Pregnancy		0.5–0.75 cc. per 100 grams of guinea-pig weight
Fetal		1.75 cc. per 100 grams of guinea-pig weight did not kill
Period of gestation, 3–8 months		0.75 cc. per 100 grams of guinea-pig weight
Normal human	Male	1.2–1.5 per 100 grams of guinea-pig weight
	Female	1.0–1.2 per 100 grams of guinea-pig weight
Menstruation	Before	0.75–1.0 per 100 grams of guinea-pig weight
	After	0.75 per 100 grams of guinea-pig weight

corpuscles, leucocytosis, changes in fibrinogen content, in enzymes and antienzymes, cholesterol and lipoid balance; clinically the changes are manifested by temperature rise, alteration of pulse rate, altered metabolism, nitrogen excretion, etc.

Numerous investigators have associated ovulation, the development of the corpus luteum, and menstruation. A similar association exists between ovulation and the development of the corpus luteum of pregnancy, and pregnancy. If fertilization takes place, the corpus luteum persists unchanged for months, and its secretion may play a prominent part in the regulation of a number of important biological alterations associated with pregnancy. If implantation does not occur the cor-

pus luteum immediately shows degenerative changes, which in turn are supposed to lead to the onset of the menstrual flow (Ruge (10), Fraenkel (11), Halban (12), Schroeder (13)). The menstrual cycle and pregnancy having many common biological alterations, one in the toxicity such as we have observed, might seem especially logical, and seems to offer conductive evidence of the association of the two conditions.

DISCUSSION AND CONCLUSIONS

Parallel with the findings of Sachs and v. Oettingen that fetal and maternal plasma occupy the opposite extremes of colloidal stability (normal plasma occupying an intermediate position), we have found that of the two substances the maternal plasma is much more toxic for the guinea-pig when intravenously injected. The fetal plasma can be injected with impunity in relatively large doses. In this regard normal plasma also occupies an intermediate position.

Both chemically and physically certain differences exist between maternal and fetal bloods. The blood lipoids differ quantitatively and even the proteins, as Naegili points out, are dissimilar. Albrecht (14), working in this laboratory, has observed that in saturating with magnesium sulphate practically no globulins are precipitated from fetal blood. Howe (15) has recently examined the differences that exist in the blood of new born calves and finds that the euglobulin and the pseudoglobulin 1 fractions are absent, but appear in the blood after the ingestion of cholostrum. Fibrinogen is present in fetal blood in increasing amounts until term (16) but the amount in the maternal blood is much greater (0.47 to 0.4: per cent as compared with 0.31 to 0.32 per cent normal, Gram (17).

Among the physical variations, the difference in surface tension is very striking, as well as the alteration in the settling time of the red blood corpuscles.

Inasmuch as the death that occurs in the guinea pig is so obviously associated with clotting phenomena, the increase in the fibrinogen content of the maternal plasma might be considered responsible. Welker found that in using serum from

rattlesnakes the serum was toxic in a degree corresponding to the fibrinogen content remaining in the serum, as soon as the fibrinogen was quantitatively removed the serum was no longer toxic. Even the serum of the maternal organism is quite toxic for the guinea pig, but this may be, as Welker found in the rattle snake, because of the fibrinogen still remaining in the serum. Adsorption by inert agents and filtration through a Berkfeld filter largely remove the factor that causes immediate death in the pig. While we can in general state that an increase in the fibrinogen content goes hand in hand with increased toxicity, it is by no means excluded that some factor associated with the fibrinogen increase rather than the fibrinogen itself, is the factor responsible.

In view of the fact that fetal plasma contains no euglobulin nor pseudoglobulin I it might be assumed that these particular proteins are associated with the toxic effect. Experiments to elucidate this particular question are now being made. Until further investigation we would draw no final conclusions concerning the particular factors responsible for the considerable differences in toxicity observed between maternal and fetal plasmas.

SUMMARY

Blood plasma obtained from pregnant women is more toxic for the guinea-pig on intravenous injection than plasma obtained from normal individuals. Human placental plasma is practically nontoxic. Similar relations are found with dog plasma obtained from pregnant dogs and from full term fetuses.

Plasma obtained during the menstrual cycle (before and after) is occasionally more toxic for guinea-pigs than normal female plasma.

The cause of death in the guinea-pig is an intravascular clotting of the capillaries of the lungs, with a resulting asphyxia, and occlusion in other capillaries as well (brain, liver, etc.). Death can be prevented if the animal is hirudinized, or if the amount of citrate simultaneously injected, is increased.

REFERENCES

(1) Sachs, H., and von Oettingen, Kj.: Münch. med. Wochenschr., 1921, **68**, 351.
(2) Hanzlik, P. J., and Karsner, H. T.: Jour. Phar. and Exp. Therap., 1920, **14**, 449.
(3) Friedberger, E.: Zeit. f. Immunität. Orig., 1913-14, **20**, 405.
(4) Nathan, E.: Zeit. f. Immunität. Orig., 1914, **23**, 204.
(5) Novy, F. G.: Jour. Infec. Dis., 1917, **20**, 536 and 657.
(6) Welker, W. H., and Marshall, J.: Jour. Phar. and Exp. Therap., 1915, **6**, 563.
(7) Webster: Montreal Med. Jour., 1897, volume **26**,
Webster, J. C.: Pelvic viscera in relation to microörganisms in health and disease, p 91.
Webster, R. E.: Strangulated umbilical herina, p. 359.
(8) Otto, A.: Vehr. des X internat. med. Congresses, Berlin, 1891, **10**.
(9) van der Velde: Wellenbewegung u. Menstrual. Jena, 1905.
(10) Ruge, Carl: Archiv. f. Gyn., 1913, **100**, 20.
(11) Fraenkel, C.: Archiv. f. Gyn., 1910, **91**, 705.
(12) Halban, J., and Kohler, R.: Archiv. f. Gyn., 1914, **103**, 575.
(13) Schroeder, C.: Archiv. f. Gyn., 1914, **101**, 1.
(14) Personal Communication.
(15) Howe, P. E.: Jour. Biolog. Chem., 1921, **49**, 115.
(16) Emmel, V. E., Levinson, S. A., Fisch, M. E.: Jour. Exp. Med., 1920, **31**, 177.
(17) Gram, H. C.: Jour. Biolog. Chem., 1921, **49**, 279.

STUDIES ON THE TOXICITY OF HUMAN BLOOD PLASMA FOR GUINEA-PIGS

II. COAGULATION TOXICITY

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I. INTRODUCTION

In our previous report (1), we have briefly mentioned the phenomena produced by the intravenous injection of human plasma into the guinea-pig which we associated with coagulation changes. It is the object of this paper to report in some detail the results of this observation, and to show briefly the relationship it has with anaphylaxis and the associated phenomena.

The theories of anaphylaxis and anaphylactic shock are so numerous that it is only possible to mention a few of them, especially those which may have any bearing on our findings. Biedl and Kraus (2), Schultz and Jordan (3) among others have shown that the peculiar and rapid fatal character of anaphylactic shock in the guinea-pig depends on the valve-like closure of the bronchioles, due partly to the special susceptibility of the bronchiolar musculature, and partly to the thick lining of these tubes in the guinea-pig, so that when contraction of the bronchi take place, the lumen is obstructed. These findings in conjunction with other explanations that may be given as to the possible cause of anaphylactic shock, presuppose the fact that the guinea-pig has been previously sensitized against a foreign substance, and then subjected to the anaphylactic shock. A similar observation was also made by Hanzlik and Karsner (4) when unspecific colloids were used in non-sensitized guinea-pigs.

Vaughan (5), Schittenhelm and Weichardt (6), and numerous other investigators have shown that various products of partial protein hydrolysis produce, on injection into normal animals, symtoms in many respects resembling anaphylactic shock. Many observers have been able to produce symtoms of anaphylactic shock in other ways, thus Richet (7), Vaughan (5), Anderson and Frost (8), by injecting into a normal animal, serum from a sensitized animal digested with the antigen, Friedberger (9), by injecting serum from a normal animal digested with specific precipitates, while Friedemann (10) used blood corpuscles sensitized with hemolysin. Just where the specific antibody and antigen union is brought about (which is cellular or humoral) is still a moot question. Some place the formation of a non-specific poison in the circulating fluids, others in the cells participating in the anaphylactic reaction. Doerr and Moldovan (11 have suggested that an immediate physical change in the blood colloids accounts for the anaphylactic reaction, a theory that is at the present under study by a number of French investigators.

Dale (12), Wells (13), as well as Besredka (14) give a detailed résumé of the various theories of anaphylaxis and anaphylactic shock. These include the anaphylatoxin theory of Friedberger (9), the apotoxin theory of Richet (7), numerous ferment theories (Jobling and Petersen (15)), the physical theory of Doerr and Moldovan (11), physical nervous theory of Besredka (14), and peptone theory of Beidl and Kraus (2). When dealing with such an intoxication as that following plasma injection in the guinea-pig these theories are of course irrelevant. Von Behring's (16) platelet theory, or the observation of Dale and Laidlow (17) as well as Hanzlik and Karsner (4), that the clumping of platelets, or conglutination of red cells and presence of thrombi in pulmonary vessels, appears to be the most logical explanation of shock taking place following an intravenous injection of foreign substance into a non-sensitized guinea-pig.

II. METHODS AND RESULTS

The method used in demonstrating this phenomenon is similar to that previously described by us when plasma from pregnant

women was injected intravenously into guinea-pigs. We wish to emphasize the fact that in these experiments, as in the others dealing with the toxicity of human plasma, we used guinea-pigs that had not been used for other purposes, and were not sensitized.

Mode of death. When approximately one half the lethal dose of human plasma is injected intravenously into a guinea-pig, the latter appears to be unaffected, and the animal lives for an indefinite period. When, however, a sublethal dose of plasma is injected intravenously, the following symptoms become manifest: The animal is at first restless; in a few seconds it becomes quiet and seems sick. Respirations are increased in frequency, the breathing seems labored and is more shallow than in the normal. A contraction of the muscles of the gastro-intestinal tract follows, and the animal as a rule passes feces, not infrequently a diarrhea may ensue. Convulsive seizures take place, in some cases appearing gradually, at other times very suddenly; the animal's head may take an oscillatory motion from left to right, then suddenly the head is thrown backward and convulsive jumping movements take place. This may last for a few seconds, and as the animal becomes exhausted it lies on one side and appears as if dead. The respirations are shallow and almost invisible, then become increased in frequency and the animal struggles to get back on its feet again. Although the animal appears to be sick and gives one the impression that it may die almost any minute, the guinea-pig may live from three to twenty-four hours, and in some cases even at a longer period makes a complete recovery.

When the guinea-pig receives a toxic dose of human plasma the phenomenon may be very similar to that here described, but as a rule the following sequence occurs: The animal immediately after the injection becomes very restless, runs about until almost exhausted, and then scratches its nose and appears to be very irritable. There then appears a contraction of smooth muscle fibers (there is a discharge of urine and feces) and a peculiar contraction of the intercostal muscles, and evidently a contraction of the bronchial muscles resulting in a peculiar respiratory grunt. Pulse and respirations are quickened and the animal may lie

over on one side and get a convulsive seizure or the latter attack may effect the guinea-pig without any warning. The head is thrown backward and the back is bent, the guinea-pig appearing in opisthotonus. These convulsive attacks may be of such a severe degree as to cause the animal to be thrown off its feet. During this attack the hind legs are straightened, and as the guinea-pig makes attempts to get on its feet again there is this added difficulty of paralysis of the hind legs; at times the front legs may also become paralyzed. After a few more shallow respiratory efforts the guinea-pig dies. Instead of the extended and rigid condition that the animal was in a few moments ago, the entire body is relaxed, the muscles are soft and the legs flexible. Upon opening the guinea-pig and examining the heart and lungs it will be seen that the heart is distended and a large blood clot fills the ventricles. The lungs are distended, firm, and elastic and feel like a semisolid piece of rubber. Occasionally, hemorrhages may be seen on the surface of the lung. When the lung is cut open the latter still retains its solid appearance, but at times a serous exudate may be squeezed from the cut surface.

Sections of the lungs of the animals that died immediately after the toxic dose of plasma was injected, were made. The tissues were fixed in Zenker's solution for twenty-four hours, then washed in distilled water for twenty-four hours and later embedded in paraffin. Sections were later stained for fibrin by using Weigart's staining method. Microscopic examination of these sections show fibrin strands and clumps of fibrin in practically all the capillaries of the lung. Occasionally red blood cells are seen engulfed in the fibrinous mass (fig. 1).

Effect of heating and filtering plasma. It was of interest to note the effect temperature changes and filtration of human plasma may have on the degree of toxicity of the latter for guinea-pigs. Sakamoto (18) has shown that lung extract, the most toxic extract of all organs, retains its toxicity for about 2 days when kept on ice and is only slightly reduced by filtration through a Berkfeld filter. This observer has also noted that when the extract is shaken with kaolin or animal charcoal the toxicity is

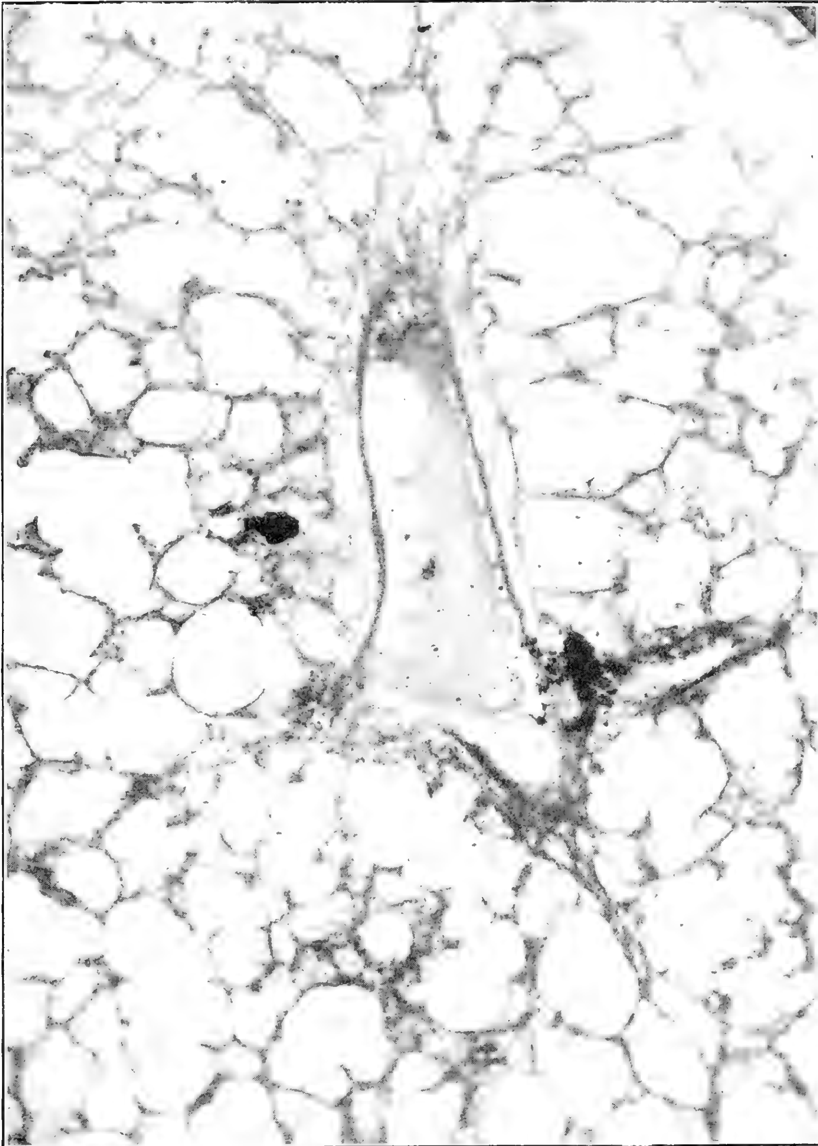

Fig. 1. Microphotograph of lung showing fibrin in the smaller vessels. There is also a marked distension of the alveoli. Zeiss × 200.

slightly reduced, but that it is not affected by a solution of egg white. When fresh normal serum is added to the extract, the toxicity is neutralized, but serum inactivated at 50°C. loses its neutralizing properties.

Our findings in many respects are similar to those of Sakamoto, and we have observed no change in the degree of toxicity when sterile human plasma is kept on ice for two days. Although Sakamoto states that the degree of toxicity is slightly reduced by filtration through the Berkefeld filter we have found that the degree of toxicity was entirely reduced to that of normal. The toxic normal dose of human plasma kills the guinea-pig in a few moments, while the same dose of the same plasma (taken from normal or pathologic cases) which has been filtered through a Berkefeld filter, does not effect the guinea-pig. In fact, the animal is as active at the end of twenty-four hours as before the injection (protocol 1).

When plasma is heated to 56°C. for twenty minutes the degree of toxicity is lessened. It will be noted that when twice the toxic dose is injected intravenously the animal remains unaffected. Although Sakamoto states that the toxicity of the lung extract is destroyed by heating at 100° for two hours and at 38° for ten hours we have not been able to use this factor because the blood plasma would clot. Heating to 56°C. for twenty minutes produces the same results as filtering the plasma through the Berkefeld filter (protocol 2).

Experiments with hirudin and citrate. One cubic centimeter of hirudin was injected intravenously into a guinea-pig and from three to five minutes later the toxic dose of the plasma was also injected intravenously. Instead of immediate death from the toxic dose, the animal remained unaffected, for at least five hours. When a dose of plasma was injected somewhat larger than the toxic dose after the animal received hirudin, the guinea-pig did not die immediately nor were marked convulsions produced, but the animal was sick and in the course of several hours it would die. Also increasing the amount of citrate does the same thing (protocol 3).

PROTOCOL 1

Effect of filtering plasma on the degree of toxicity for guinea-pigs

WEIGHT OF GUINEA-PIG	DOSE OF PLASMA INJECTED PER 100 GRAMS OF GUINEA-PIG WEIGHT	AMOUNT OF PLASMA INJECTED	RESULT	DOSE OF PLASMA INJECTED PER 100 GRAMS OF GUINEA-PIG WEIGHT	AMOUNT OF PLASMA INJECTED AFTER FILTRATION THROUGH A BERKEFELD FILTER	RESULT
grams	*cc.*	*cc.*		*cc.*	*cc.*	
250	1	2.5	Immediate death	1	2.5	Alive at end of 24 hours
235	1	2.35	Immediate death	1	2.35	Alive at end of 24 hours

PROTOCOL 2

Effect of heating plasma on the degree of toxicity for guinea-pigs

WEIGHT OF GUINEA-PIG	DOSE OF PLASMA INJECTED PER 100 GRAMS OF GUINEA-PIG WEIGHT	AMOUNT OF PLASMA INJECTED	RESULT	DOSE OF PLASMA INJECTED PER 100 GRAMS OF GUINEA-PIG WEIGHT	AMOUNT OF PLASMA INJECTED AFTER HEATING TO 56° FOR 20 MINUTES	RESULT
grams	*cc.*	*cc.*		*cc.*	*cc.*	
260	1	2.6	Immediate death	2	5.2	Alive at end of 24 hours
200	1	2.0	Immediate death	2	4.0	Alive at end of 24 hours

PROTOCOL 3

Effect of previous intravenous injections of hirudin on the degree of toxicity of human plasma for guinea-pigs

WEIGHT OF GUINEA-PIG	DOSE OF PLASMA INJECTED PER 100 GRAMS OF GUINEA-PIG WEIGHT	AMOUNT OF PLASMA INJECTED	RESULT	AMOUNT OF HIRUDIN INJECTED	AMOUNT OF PLASMA INJECTED	RESULT
grams	*cc.*	*cc.*		*cc.*	*cc.*	
240	1	2.4	Immediate death	1	2.4	Alive at end of 5 hours
275	1	2.75	Immediate death	1	2.75	Alive at end of 6 hours

III. DISCUSSION

In solving this problem, the method of approach would be by the process of elimination. If previous sensitization of an animal by a foreign substance causes a liberation of certain antibodies in the blood stream, and a subsequent injection of the same or other foreign substance produces death by virtue of the antigen combining with the antibody in the blood vascular system, then this explanation for coagulation toxicity does not hold true, since only one foreign substance is injected and produces death. Doerr and Moldovan (11) suppose that the effect of the antigen, in contact with the antibody of the sensitized animal, alters the state of aggregation of the colloids in the blood plasma, and induces changes of surface tension, which are responsible for the effect on the plain muscle and other tissues. None of the supposed theories that have been formulated to explain anaphylaxis or anaphylactoid shock can be used wholly or partially in explaining our phenomenon of coagulation toxicity, as long as sensitized animals are used for these experiments.

Hanzlik and Karsner have demonstrated that by the intravenous injection of various colloidal agents such as agar, congo red acacia, and other products they were able to produce inflation of the lungs very similar to that found in true anaphylactic shock.

v. Behring's platelet theory seems of considerable interest in the mechanism of death with which we are dealing, and this explanation comes closer to the actual state of affairs than do most of the other theories. v. Behring (16) was of the opinion that some disturbance of the platelets, endogenous or exogenous in character, results in a clumping of the platelets in the smallest blood vessels forming capillary emboli and symptoms of anaphylactic shock. Freund (19) has shown that by injecting fresh serum intravenously into a rabbit, the animal is killed instantly. This observer has also shown that when fresh serum is injected into cats, the temperature drops, the respiratory center becomes involved, and there is a "shock effect" on the heart with a fall in blood pressure. Freund is of the opinion that sudden death following an intravenous injection of a foreign protein is due to broken

down platelets which disturb the cellular elements in the blood. The physical and chemical state of the blood platelets, may also become altered and these in time may become toxic to or occlude the smaller blood vessels. Freund and Gottlieb (20) have also noted that the breaking down of cellular elements can influence the autonomic function very markedly. In pregnancy Neu and others have demonstrated that vasoconstrictor substance affects the vascular system more so than in normal persons. This observation has also been made by Hanzlik and Karsner, who have shown that any foreign substance that may be injected intravenously into an animal, may have the power of obstructing smaller blood vessels, and thereby produce a phenomenon similar to that described by v. Behring. Many of these inorganic colloids have the property of causing a mutual precipitation when they are mixed with a solution of protein, and this probably takes place within the blood vessels when inorganic colloids are injected intravenously. It is possible that the agglutination of corpuscles and the formation of thrombi which takes place within the capillaries on the injection of bacteria, as in pseudoänaphylaxis (Herzfeld and Klinger (21)) is the result of the bacterial extracts from these organisms forming an active ingredient in the process of coagulation. It will be observed that none of the theories mentioned, or those enumerated in the various monographs and discussions on the subject deal with fibrinogen as possible factor in anaphylaxis or associated phenomena.

That the stability of the colloids in the blood stream is to be considered an important factor in the study of hypersensitiveness has recently been emphasized by Sachs (22). The fibrinogen fraction of the blood contrasted to the albumen fraction is colloidally very unstabile. Sachs and von Oettingen (23) have shown that blood plasma from normal human pregnant cases, from normal individuals, and from fetal cases will flocculate in the order mentioned. The fibrinogen in the maternal blood plasma is very labile and therefore flocculates readily, while the fibrinogen fraction of the fetal blood plasma seems more stabile; the plasma may show a faint turbidity when subject to a variety of procedures (heat, alcohol, etc.) but no marked flocculation as is seen in the

maternal plasma. We have been able to corroborate these observations and have also noted that there was a difference in the degree of flocculation and turbidity between adult male and female plasma. There was a tendency of the latter plasma to flocculate sooner than the former, the probable reason being that the female plasma contains a higher percentage of fibrin than does the male plasma. This evidence, along with the observations of Fahraeus (24), Doerr (25), Linzenmeier,(26) Plaut and Pagniez (27) and others that the rate of sedimentation of the red blood cells in pregnancy as well as in other conditions depends upon the fibrinogen content of the blood plasma, is further proof of the importance of fibrinogen fraction. Because of the fact that the degree of toxicity for guinea-pigs is higher in those conditions in which the fibrinogen content is increased (pregnancy, acute stage of pneumonia, severe heart cases), and that we have also been able to observe fibrin strands and clumps in the smaller vessels and capillaries, we can not escape the conclusion that the phenomenon of coagulation toxicity when plasma is injected in guinea-pigs is associated with the quantitative fibrinogen content of the blood plasma injected. The mechanism causing death in the guinea pig no doubt is due to emboli in the terminal vessels, particularly in the lungs, heart, and brain, and possibly in the liver.

We have demonstrated that when an agent such as hirudin is injected intravenously into a guinea-pig with the purpose of preventing an intravascular coagulation, the toxic dose of the blood plasma will not kill the animal. This at once shows that the mechanism of death is due to a coagulation taking place within the blood vessels. By filtering the blood plasma through a Berkefeld filter the toxicity can be reduced, and heating the plasma to 56°C. for twenty minutes produces the same results. It is possible that the Berkefeld filter by absorption prevents some of the colloidal fibrinogen from passing through the filter, and that heating either disperses the fibrinogen or brings about absorptive aggregates with smaller colloidal particles with a lessening of potential toxicity. We have observed that when a clot forms in the plasma during centrifuging the blood, or forms when the plasma stands at room temperature for any length of time, the

toxicity of the plasma is reduced, indicating that the fibrinogen is an active factor in determining the degree of toxicity. If we assume that "colloidoclasis" is one of the essential phenomena associated with anaphylaxis, a supposition that has found many adherents among French scientists (Lumiere (28), Kopaczewski (29) etc.) it is apparent that any alteration in the balance of the plasma colloids will be of profound importance, particularly if the colloidal balance is altered in the direction of a lessened stability. In such states it is obvious that anaphylactoid phenomena should be easily induced and relatively frequent. We might be warranted in assuming that (in view of the relative unstability of the fibrinogen fraction), toxic manifestations should become clinically apparent in diseases associated with an increased fibrinogen content of the plasma. Whether eclampsia and the toxemias of pregnancy are to be grouped here is not determined, but the frequency of capillary thrombosis in eclampsia might seem to favor this idea. So, too, the whole group of manifestations that French clinicians associate with the 'rheumatoid state' (manifestations in the joint membranes, and other serous cavities, in the eye, skin and intestinal tract, etc.) might be investigated profitably from this point of view. In general one might find some warrant for assuming that the fibrinogen fraction being unstable, there would be a tendency to symptoms analogous to anaphylactic shock in those patients who have a high fibrinogen content, but one must remember that in a diseased condition, especially where catabolic processes take place at a rapid rate, there is a tendency for the organism to adjust the balance or to neutralize the factor which causes this disturbance.

REFERENCES

(1) Levinson, S. A.: This Journal.

(2) Biedl, A., and Kraus, R: Wiener k. Woch., 1910, **23**, 385.

(3) Schultz, W. H., and Jordan, H. E.: Jour. Phar. and Exp. Therap., 1911, **11**, 375.

(4) Hanzlik, P., and Karsner, H. T.: Jour. Phar. and Exp. Therap., 1920 **14**, 463.

(5) Vaughan, Victor C.: Jour. Infect. Dis., 1907, **4**, 476.

(6) Schittenhelm, H., and Weichardt, W.: Münch. med. Wochen., 1912, **59**, 67.

(7) Richet, Ch.: L'Anaphylaxie, Paris, 1912.
(8) Anderson, J. F., and Frost, W. H.: Hyg. Lab. Bulletins, Washington, 1910, No. 64.
(9) Friedberger, E.: Zeit. f. Immunitat. "Orig.," 1909, **2**, 208.
(10) Friedemann, Ulrich: Jahersb. uber d. Ergeb. d. Immunitat., orig., 1910, **6,** Abt. i, 31.
(11) Doerr, R., and Moldovan, J.: Biochem. Zeit., 1912, **41,** 27.
(12) Dale, H. H.: Jour. Phar. and Exp. Therap., 1913, **4,** 167.
(13) Wells, H. G.: Physiological Reviews, 1921, **1,** 44.
(14) Besredka, A: Anaphylaxie et Antianaphylaxie, 1917, Masson et Cie, Paris.
(15) Jobling, J. W., and Petersen, W.: Jour. Exp. Med., 1914, **20,** 468.
(16) von Behring, E.: Deut. Med. Wochen., 1914, 40, 1833.
(17) Dale, H. H. and Laidlow, P. P.: Jour. Phys., 1919, **52,** 355.
(18) Sakamoto, E.: Zeit. f. Immunitat., Orig., 1921, **32,** 1.
(19) Freund, H.: Archiv. Exp. Path. and Phar., 1921, **91,** 272.
(20) Freund, H., and Gottlieb R.: Münch. med. Wochenschr., 1921, **68,** 383.
(21) Herzfeld, E. and Klinger, R.: Schw. Med. Wchr. 1920, **1,** 567.
(22) Sachs, H.: Kolloidzeitschr. 1919, **24,** 113.
(23) Sachs, H., and v. Oettingen, K.: Münch. med. Wochenschr., 1921, **68,** 351.
(24) Fahraeus, R.: Acta Medica Scand. 1921, **55,** 3.
(25) Doerr, R.: Schweiz. med. Wochen., 1921, **51,** 937.
(26) Linzenmeier, G,: Med. Kl., 1920, **16,** 439.
(27) Pagniez,P.: Presse Medicale, 1921, **29,** 405.
(28) Lumiére A.: "Antianaphylactic Shock and Colloioclasis." Press med., 1921, **11,** 960.
Lumiére, A.: Colloidal Shock. Paris Med., 1921, **11,** 445.
(29) Kopaczewski, M. W.: Paris Med., 1921, **11,** 379.

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