# EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

## I. Type Experiments with Known Antigens—a Bacterial Hemotoxin (Megatheriolysin), the Pneumococcus, and Poliomyelitic Virus.

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There are two generally recognized prerequisites to any attempt at the production in animals of an antiserum to combat an infectious disease. These are, needless to state, the isolation and the successful cultivation *in vitro* of the disease's causative agent. The difficulties here encountered in the case of some common and important maladies have absolutely held up all advances toward specific treatment.

The possibility of immunizing animals by direct injections of infected human tissues is one that must have occurred to many minds; and some attempts to employ the method were made prior to the general recognition that tissue itself acts to engender antibodies highly injurious to the species from which it is derived. With that recognition the method was given up, and it has not been revived. Yet there are noteworthy instances, such as the Pasteur treatment for rabies, which prove that tissue may contain an infectious agent in sufficient quantity to act as a practical antigen. And living tissues are superior in one respect to all other culture media, since organisms flourishing in them possess those pathogenic characters, loss of which in the test-tube sometimes limits, or indeed prevents, the development of antibodies in animals injected with cultures.

It has seemed to us possible that an antiserum produced by the injection of infected tissues might be rendered fit for employment in the species from which the tissues are derived by submitting it to a process of selective absorption, or exhaustion, with suspensions of

normal tissues. Such treated serum would, supposedly, be deprived of injurious tissue antibodies while retaining those directed against the infectious agent. A number of difficulties at once suggest themselves. It is well known that the absorption of antibodies is to a remarkable degree selective; and yet may not the repeated absorption of a polyvalent serum with tissue cells weaken notably the general content in antibodies? Will not toxic elements develop as a result of the incubation *in vitro* of mixtures of serum and tissue? And even should this not occur, is it really possible by the method of absorption to rid a serum of its anti-tissue potency so far as to render it harmless *in vivo*? May not the success of the absorptions be dependent on the employment of tissues derived from the same organ or organs used in the immunization? And finally, will not the method after all be more tedious than practical?

Some of these questions can be answered out of present knowledge. The recent work of many investigators on anaphylatoxins has shown that toxic elements are indeed engendered in incubated mixtures of tissue and its antibody, but only when complement is present.[1] In our selective absorptions inactivated serum could and should be used. The amount of tissue required to exhaust a serum of even high anti-tissue titer need not be great, since, as has been repeatedly shown in the typical case of red cells, many times the minimum hemolytic unit of amboceptor or agglutinin may be taken from a serum by a single unit of antigen. Furthermore, in the case of most sera, one can hope to use red corpuscles instead of tissues of the precise sort employed in the immunization. For much previous work has clearly demonstrated that only in certain special instances are specific cytotoxins the result of tissue injections.[2] Usually hemolysins, hemagglutinins, and serum precipitins alone develop.

In the work to be described our aim has been to determine the fundamental point, whether sera obtained by the immunization of animals with infected tissue of another species can, by the method of absorption, be rendered available for therapeutic use in the last mentioned species. No attempt has been made to devise practical

[1] See, for example, Friedberger, E., *Z. Immunitätsforsch.*, 1910, iv, 636.

[2] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

methods of absorption, to determine the least amount of tissue that will exhaust a serum, etc. For the purpose of type experiments sera have been selected containing different kinds of antibodies directed in all except one case—that of the chicken tumor—against pathogenic agents already isolated and well studied.

*Selective Absorption Applied to an Antitoxic Serum.*

The simplest case, that of an antitoxic serum, was first taken up. In view of the possible importance of "anaphylatoxins" engendered in the incubated mixture of serum and tissue as above mentioned, it seemed best to work with the animals most susceptible to such poisons; namely, guinea pigs. Furthermore, it was desirable to employ, if possible, a toxin of which the neutralization with antitoxin could be observed *in vitro* as well as *in vivo*. Both conditions are met by the hemolysin produced by *Bacillus megatherium* and first studied by Todd.[3] Todd demonstrated that the lysin is a true toxin, against which an antitoxin can be readily produced in guinea pigs, rabbits, and goats. His work has been fully confirmed.[4] *Bacillus megatherium* produces its toxin only when grown *in vitro* under special conditions, and although the toxin will kill guinea pigs, the organism itself is practically non-pathogenic. For this reason infected tissues could not be obtained for the immunizations required by our plan. Normal tissues mixed with the toxin might perhaps have been used, but the latter by itself, when injected locally, causes a violent inflammatory reaction. It was decided, on this account, to inject the toxin and the tissue used for immunization at separate sites and at different times.

*Characters of the Megatheriolysin.*—Three strains of *B. megatherium* were obtained from the American Museum of Natural History through the kindness of Professor Winslow. Only one produced any noteworthy hemolysin when cultivated in the special bouillon recommended by Todd.[3] This produced a lysin of such strength that 1 cc. of the Berkefeld filtrate of a 7 day culture, when in-

[3] Todd, C., *Lancet*, 1901, ii, 1663; *Tr. Path. Soc. London*, 1902, liii, 196.

[4] Dreyer, G., and Blake, J., *Lancet*, 1904, ii, 408. Craw, J. A., *Proc. Roy. Soc. London, Series B*, 1905, lxxvi, 179. Vincent, H., *Compt. rend. Soc. biol.*, 1909, lxvii, 195.

jected intravenously into a 400 gm. guinea pig, regularly caused intense hemolysis and hemoglobinuria, with death in a few hours at most. Intraperitoneally 2 cc. produced death quite as rapidly from diffuse petechial hemorrhages, first into the mucosa of the small intestine, and thence into the lumen of the intestine, which became distended with fluid blood. The uterus, large intestine, and stomach sometimes showed petechiæ, usually scattering. Despite the striking local lesions there was no evidence of intravascular hemolysis and never any hemoglobinuria, even when death occurred slowly. When given subcutaneously the lysin caused a wide area of necrosis.

The strain of *B. megatherium* furnishing the lysin was cultivated in quantity in Todd's medium, and at the end of 7 days the culture fluid was centrifuged and passed through a Berkefeld filter, after which the filtrate was tested for sterility, tubed, the tubes sealed with paraffin, and stored in the dark at 2–3°C. Under these conditions the lysin was found to retain practically all its activity for months, a great advantage, since portions of the same filtrate could be used both for the immunization of animals and for tests of the antisera that they yielded.

## *Immunization of Animals.*

The lysin is most injurious to guinea pigs, yet large amounts are ill tolerated by rabbits or goats. The normal serum of both these animals has some slight neutralizing activity for the lysin, as shown by the ability to prevent hemolysis of guinea pig corpuscles. Attempts were made at first to immunize rabbits. A number of these animals were given six intraperitoneal injections at 6 day intervals of defibrinated guinea pig blood plus suspensions of ground liver and spleen, followed 2 days later by intraperitoneal injections of 1 cc. of the megatheriolysin. The animals were bled 7 days after the last lysin injection. They stood the immunization badly; all lost weight, and several died. Nevertheless, as will be seen, the survivors elaborated a well marked antitoxin.

A goat was immunized by separate subcutaneous injections of tissue and lysin according to the method just outlined. The finely ground liver, spleen, and kidney of guinea pigs, and the defibrinated blood were mixed and used. The amount of megatheriolysin given was gradually increased from 0.5 to 10 cc., diluted always with salt solution. This caused boggy swellings which were slow to subside. 7 days after the last of the six lysin injections the goat was bled, and the serum was tubed without the addition of preservatives and left in the ice box. The rabbit serum was similarly treated.

## *Method of Exhaustion with Red Cells.*

The serum was inactivated at 56°C. for ½ hour just prior to use. Guinea pig red cells taken into citrate were thrice washed in ¼ per cent gelatin Locke's solution under aseptic conditions. In ordinary salt solutions guinea pig corpuscles

are prone to break down when washed; but the addition of a little gelatin to the washing fluid will prevent this.[5] After the last washing the cells were packed in graduated tubes, their bulk was noted, all possible fluid pipetted off, and the serum to be exhausted poured on, the tube corked, inverted, and gently shaken to suspend the cells. The suspension was now warmed to 38°C. in the water bath, incubated, centrifuged at high speed, and the serum transferred to a fresh lot of cells. The period of incubation varied with the degree of agglutination of the cells. When they fell out promptly into a solid mass such as could not be broken up without hemolysis, incubation for more than a few minutes was manifestly useless. But often after one or two absorptions the cells tended to remain in suspension, and the incubation period was lengthened to an hour. Finally, when the suspended cells showed no trace of agglutination, incubation was continued for as long as 2 hours in some instances.

No preservative was added to the mixture of serum and cells, but great care was taken to assure its sterility. The special corks for centrifuge tubes, elsewhere described,[5] were an aid in this connection. After the last absorption cultures were regularly taken. These showed an entire absence of infection, which may perhaps be attributed as much to the frequent centrifugation at high speed to which the serum was subjected as to our technique.

With repeated absorption there was a slight unavoidable loss of serum, and a slight dilution of it also occurred, owing to the remnant of gelatin Locke's solution introduced with the cells. In testing the relative potency of the unexhausted and exhausted serum usually no correction was made for this dilution of the latter.

## *Specimen Experiments—Rabbit Serum.*

### *Experiment 1.*

For this and all the subsequent tests the sera were inactivated at 56°C. for ½ hour. Whenever the period of incubation of the *in vitro* mixture is not specifically mentioned it was for 2 hours at 38°C. Readings were made after the tubes had stood over night in the ice box.

*Hemolytic Activity of the Megatheriolysin.*—This was determined as follows: 0.25 cc. of megatheriolysin in graded dilutions + 0.25 cc. of 0.9 per cent sodium chloride + 0.25 cc. of a 5 per cent suspension of washed guinea pig red cells.

| Lysin strength* | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 |
|---|---|---|---|---|---|---|---|
| Hemolysis | C. | C. | C. | C. | +++ | Ft. Tr. | 0 |

* The lysin strength is expressed in terms of the undiluted material.

*Antilytic Titer of Normal Serum.*—The antilytic titer was tested of the inactivated sera of four normal rabbits, as contrasted with that of an animal repeatedly injected with megatheriolysin and guinea pig tissues. Mixtures were made of

[5] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

the sera in graded dilutions with a fixed amount of megatheriolysin, guinea pig red cells were added, and incubation was done. The amount of lysin in each tube was more than eight times that necessary under ordinary conditions for complete hemolysis of the corpuscles (*vide supra*). Subsidiary tests which need not here be given in detail showed that rabbit serum exercised its whole neutralizing effect on the lysin practically at once when mixed with it at room temperature. Consequently no interval was allowed to elapse before the red corpuscles were added.

0.25 cc. of rabbit serum in graded dilutions + 0.25 cc. of $\frac{1}{8}$ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

| Serum. | | Serum dilution. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |
| Normal. | A..... | ++ | +++ | ++++ | Alm. C. | C.(?) | C. | C. | C. |
| | B..... | + | + | ++ | +++ | ++++ | Alm. C. | " | " |
| | C..... | + | + | ++ | ++ | +++ | " " | " | " |
| | D..... | + | ++ | ++ | ++++ | ++++ | " " | " | " |
| Immune..... | | 0 | 0 | 0 | Tr. | +++ | " " | " | " |

It is evident from this experiment that the normal rabbit sera possessed some power to prevent destruction of corpuscles by the megatheriolysin. But the immune serum conferred at least eight times as much protection, as shown by comparing its effect, when diluted, with that of the concentrated normal sera. The failure of the immune serum to protect to the same proportional degree in the higher dilutions is attributable to the presence in megatheriolysin of several lytic components[3] against all of which doubtless the antiserum had not the same relative activity.

*Anti-Guinea Pig Titer of the Immune Serum.*—This was well marked as a result of the repeated injection of the immunized rabbit with guinea pig tissue. A precipitin was present effective against dilutions of guinea pig serum up to and including 1:256 when an equal bulk of the concentrated immune serum was mixed with it. Tests for hemolysis and agglutination were made as follows:

0.25 cc. of serum dilution + 0.25 cc. of 1 in 10 guinea pig complement + 0.25 cc. of 5 per cent guinea pig red cells.

| | Serum dilution. | | | | | | | | | | | Complement + red cells + 0.25 cc. of salt solution. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1,024 | |
| Hemolysis..... | 0 | + | +++ | ++ | ± | ± | Tr. | Tr. | Ft. Tr. | 0 | 0 | 0 |
| Agglutination.... | C. | C. | C. | C. | C. | C. | ++++ | ± | Tr. | 0 | 0 | 0 |

The low hemolytic titer was doubtless due in part to serum precipitation which took place in the mixtures, for complement is absorbed during such precipitation, as is well known. But it is also explained by the close biological relationship of rabbit and guinea pig which renders difficult the production of antibodies in the one against the other. The fact should also be recalled that specific hemolysins as a rule act but weakly when complemented with serum from the species furnishing the test cells. Yet it was deemed best to use such complement in our tests, since it would be the only one present during *in vivo* experiments.

*Selective Absorption of the Anti-Guinea Pig Elements.*—3 cc. each of the four normal sera and the immune serum above mentioned, all inactivated, were mixed respectively with 1.5 cc. of sedimented guinea pig red cells, incubated for 1 hour, and centrifuged, and the serum was transferred to a fresh portion of red cells. In the case of Normal Sera C and D only 0.5 and 0.65 cc. respectively of red cells were employed in the second absorption, while for the others 1.5 cc. were used as before. Incubation again was for 1 hour. In the first mixture of immune serum and cells a moderate agglutination was to be seen. None of the other mixtures ever showed the least trace of clumping.

Tests were made after the second absorption to determine how completely hemolysin and agglutinin had been removed from the immune serum. No trace of either was found. In view of these results tests to show whether the normal rabbit sera had been completely exhausted were deemed unnecessary, since such sera when untreated are almost devoid of antibodies for guinea pig red cells.

*Antilytic Titer of the Treated Immune Serum.*—0.25 cc. of immune serum in graded dilutions + 0.25 cc. of $\frac{1}{5}$ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

| Serum. | Serum dilution. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |
| Untreated | 0 | 0 | 0 | Tr. | +++ | Alm. C. | C. | C. |
| Exhausted | 0 | 0 | 0 | +++ | Alm. C. | C. | " | " |

The exhaustion with red cells had but little diminished the antimegatheriolytic titer of the serum.

*In Vivo Tests of the Neutralization of Megatheriolysin with Exhausted Serum.*—A number of mixtures containing 1 cc. of undiluted megatheriolysin with 0.75 cc. of serum or salt solution were incubated 2 hours, and 1.5 cc. of each were injected directly into the ear vein of a guinea pig. The method of injection has been described elsewhere.[6] The long period of incubation was unnecessary, since neutralization of the lysin is, as has been stated, practically instantaneous at room temperature.

[6] Rous, P., *J. Exp. Med.*, 1918, xxvii, 459.

| Guinea Pig No. | Weight. | Mixture used for injection. | Result. |
|---|---|---|---|
| | *gm.* | | |
| 1 | 350 | Exhausted immune serum + lysin. | Remained well. |
| 2 | 350 | Untreated " " + " | Died immediately after injection. |
| 3 | 375 | Exhausted normal serum (No. 1) + lysin. | Died 6 hrs. later; intense hemolysis; hemoglobinuria. |
| 4 | 350 | Exhausted normal serum (No. 2) + lysin. | Died 10 hrs. later; intense hemolysis; hemoglobinuria. |
| 5 | 375 | Exhausted normal serum (No. 3) + lysin.* | Died 40 hrs. later; intense hemolysis; hemoglobinuria. |
| 6 | 375 | Exhausted normal serum (No. 4) + lysin. | Died 10 hrs. later; intense hemolysis; hemoglobinuria. |
| 7 | 350 | Salt solution + lysin. | Died 13 min. later; intense hemolysis; at autopsy characteristic lesions. |
| 8 | 350 | " " + " | Died 12 hrs. later; intense hemolysis; hemoglobinuria. |

* Part of injection material lost.

All the animals that succumbed, except No. 2, showed the lesions already described as characteristic of the megatheriolysin. In this case death was practically immediate, and there were no gross lesions except an almost complete intravascular hemagglutination, caused, of course, by the untreated immune serum, and without doubt the cause of death.

## *Experiment 2.*

Another less comprehensive experiment with immune rabbit serum will be briefly quoted, since in addition to showing the antitoxic power of the exhausted serum it affords an interesting contrast between the lesions caused by the megatheriolysin, as such, and mixtures of the lysin with unexhausted serum.

The serum of an immunized rabbit was incubated as already described with two successive batches of washed guinea pig red cells. *In vitro* tests showed that the hemolysin and agglutinin were thus removed, whereas the antilysin was practically as strong as ever. The following mixtures were now made: (*a*) 2 cc. of megatheriolysin + 1.25 cc. of serum or salt solution; (*b*) 4 cc. of megatheriolysin + 2.35 cc. of serum or salt solution. After 2 hours incubation at 38°C. the whole of each mixture was injected into the peritoneal cavity of a guinea pig.

| Guinea Pig No. | Weight. | Mixture (*a*). Lysin + | Result. |
|---|---|---|---|
| | *gm.* | | |
| 9 | 480 | Exhausted immune serum. | Remained well; no anemia. |
| 10 | 480 | Untreated " " | Died after 48 hrs.; extreme anemia; hemoglobinuria. |
| 11 | 480 | Salt solution. | Died after 4½ hrs.; lesions characteristic of the megatheriolysin. |
| | | Mixture (*b*). Lysin + | |
| 12 | 500 | Exhausted immune serum. | Remained well; no anemia. |
| 13 | 500 | Untreated " " | Died after 48 hrs.; progressive anemia; hemoglobinuria. |
| 14 | 520 | Salt solution. | Died after 1½ hrs.; lesions characteristic of the megatheriolysin. |

The hemoglobin percentage in the blood of the surviving animals was followed for some days.

At autopsy the animals receiving megatheriolysin + salt solution presented the findings already described as characteristic after intraperitoneal injections. The small intestines were distended with blood from many fine hemorrhages into the mucosa. Other hemorrhages were present in the walls of the large intestine and stomach. The blood remaining in the vessels was unclotted, greatly concentrated, but unhemolyzed and unagglutinated. There was never any hemoglobinuria. The lesions from mixtures of the lysin with unexhausted serum were entirely different, being those characteristic of a serum hemolysin. A severe progressive anemia developed, accompanied by hemoglobinuria, and the blood specimens showed marked agglutination and many shadows. At autopsy there was no trace of intestinal hemorrhages such as result from the megatheriolysin. The spleen was greatly enlarged and crowded with phagocytes filled with red cells. There were also scattered ecchymoses on the pleuræ, diaphragm, and parietal peritoneum—a lesion never observed as the result of the megatheriolysin but commonly produced by a serum hemolysin. The conclusion is unavoidable that the animals had been saved from the action of the megatheriolysin only to succumb to that of the serum hemolysin.

## *Specimen Experiment—Goat Serum.*

### *Experiment 3.*

A freshly prepared lysin was employed for the work. Its titer diminished so little in the course of the 6 weeks during which a goat was repeatedly injected with it that tests made of the neutralizing activity of the serum of the animal before and after the immunization can be directly compared.

*Hemolytic Activity of the Lysin.*—0.2 cc. of megatheriolysin in graded dilutions + 0.2 cc. of 5 per cent guinea pig red cells.

| Hemolysis. | Lysin strength. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 |
| At first test..... | C. | C. | C. | C. | C. | Alm. C. | +++ | Tr. |
| 6 wks. later..... | " | " | " | " | Alm. C. | " " | + | " |

*Antilytic Titer of the Goat Serum before and after Immunization.*—These tests were made at the same time as those of the megatheriolysin just quoted. 0.25 cc. of goat serum in graded dilution + 0.25 cc. of $\frac{1}{8}$ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red blood cells.

| Serum. | Serum dilution. | | | | | | | | | Whole strength serum + red cells + salt solution. |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | |
| Prior to immunization..... | +++ | +++ | ++++ | Alm. C. | Alm. C. | Alm. C. | C. | C. | C. | 0 |
| After immunization (6 wks. later)..... | 0 | 0 | 0 | ± | C. | C. | " | " | " | |
| Serum of a normal goat (control)..... | Alm. C. | C. (?) | C. | C. | " | " | " | " | " | |

The serum of the normal control was obtained and tested at the same time as the immune serum.

Plainly the immunization with megatheriolysin had increased the antilytic titer of the goat serum.

*Anti-Guinea Pig Elements and Their Selective Absorption.*—Preliminary tests showed that the serum of the immunized goat contained powerful antibodies for guinea pig red cells, as would naturally follow from the repeated injection of the animal with tissues of this species. An attempt was made to absorb the antibodies from a portion of the serum, and the titer of the exhausted specimen was then compared with that of an untreated portion. The normal control serum was not submitted to absorption because it was found to be harmless to guinea pigs when given in the doses required by our experiments.

For the purposes of exhaustion 17 cc. of the immune serum were incubated for 1 hour with 5 cc. of washed guinea pig red cells, the mixture was centrifuged, and

the serum transferred to more red cells and incubated again. This was done five successive times. In the first mixtures the red cells were only moderately agglutinated, but in the later ones clumping became much more marked owing doubtless to the absorption of proagglutinoids, which at first had hindered agglutination.

| Mixture. | Hemagglutination. |
|---|---|
| 17 cc. of serum + 5 cc. of red cells incubated 1 hr. and | Moderate. |
| serum transferred to 4.25 cc. of red cells, incubated 1 hr. and | " |
| " " " 7.6 " " " " " 1 " " | Strong. |
| " " " 7.3 " " " " " 1 " " | Almost massive. |
| " " " 5.2 " " " " " 1 " | Moderate. |

Only 13.5 cc. of serum were finally recovered. The diminution in volume was due to a retention of the fluid amidst the agglutinated red cells. The latter, when clumped by the serum, occupied a greater space than when simply sedimented in salt solution.

A comparison was now made of the hemolytic and agglutinative titer of the exhausted and unexhausted immune serum, and of the normal control serum. 0.2 cc. of inactivated goat serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent guinea pig red cells were used.

| | Serum dilution. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1,024 | 1/2,048 | 1/4,096 |
| Serum. | Hemolysis. | | | | | | | | | | | | |
| Untreated normal..... | +− | Tr. | Tr. | Ft. Tr. | 0 (?) | 0 | 0 | 0 | 0 | 0 | | | |
| Untreated immune..... | Ft. Tr. | ++− | ++ | + | Tr. | Ft. Tr. | Ft. Tr. | − | 0 | 0 | | | |
| Exhausted immune..... | No hemolysis. | | | | | | | | | | | | |
| Serum. | Agglutination. | | | | | | | | | | | | |
| Untreated normal..... | No agglutination. | | | | | | | | | | | | |
| Untreated immune..... | +++ | + | ++ | C. | C. | C. | C. | C. | C. | ++ | + | Tr. | 0 |
| Exhausted immune..... | +++ | +++ | +++ | ++ | + | ± | 0 | 0 | 0 | 0 | | | |

Agglutination was read in the same mixtures as hemolysis. With rabbit complement the untreated immune serum was found to be far more hemolytic than with guinea pig complement as here shown. Precipitation was observed in the hemolytic mixtures containing immune serum in dilutions up to 1: 64, and to this is attributable the Neisser-Wechsberg phenomenon observed in the hemolytic tests of the untreated specimen.

*Antilytic Titer of the Treated Immune Serum.*—0.25 cc. of serum in graded dilutions + 0.25 cc. of $\frac{1}{8}$ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

| Serum. | Serum dilution. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 |
| Untreated immune..... | 0 | 0 | 0 | ± | C. | C. | C. | C. | C. |
| Exhausted " ..... | 0 | 0 | 0 | ++ | " | " | " | " | " |
| Untreated normal..... | Alm. C. | C. (?) | C. | C. | " | " | " | " | " |

The repeated absorption of the immune serum with large amounts of red cells (29.35 cc. of the latter in all, as against only 17 cc. of serum) was found scarcely to affect the antimegatheriolytic titer, which remained more than eight times that of the normal serum.

## *In Vivo Tests of the Neutralization.*

(*a*) *Intraperitoneal Injections.*—Mixtures of 2 cc. of megatheriolysin (or salt solution) with 2.5 cc. of serum (or salt solution) were incubated 1 hour at 38°C and injected into a number of guinea pigs of 400 gm. weight.

| Guinea Pig No. | Mixture injected. | Result. |
|---|---|---|
| 15 | Normal serum + salt solution. | Remained well; no anemia. |
| 16 | 0.9 per cent salt solution + lysin. | Died after 1¼ hrs. with characteristic lesions. |
| 17 | Normal serum + lysin. | " " 12 " " " " |
| 18 | Unabsorbed immune serum + lysin. | " " 53 " " progressive anemia and hemoglobinuria. |
| 19 | Absorbed immune serum + lysin. | Remained perfectly well save for a slight anemia soon repaired. |

The order of injection was as follows: Nos. 15, 17, 19, 18, 16.

The findings at autopsy were similar to those of Experiment 2 with rabbit serum, as already described. All the animals that died, save No. 18, showed

lesions characteristic of the action of the megatheriolysin. In the guinea pig mentioned the lesions were of a different sort, being such as are caused by a specific hemolysin; and no trace of injury from the megatheriolysin was to be seen.

(*b*) *Intravenous Injections.*—Mixtures of 1 cc. of lysin (or salt solution) with 0.8 cc. of serum (or salt solution) were incubated 1 hour at 38°C., and 1.5 cc. of each mixture were injected into the ear vein of a guinea pig.

| Guinea Pig No. | Mixture injected. | Result. |
|---|---|---|
| 20 | Normal serum + salt solution. | Remained well; no anemia. |
| 21 | " " + lysin. | Died after 42 hrs. with progressive anemia and hemoglobinuria. |
| 22 | Salt solution + " | Died after 47 hrs. with progressive anemia and hemoglobinuria. |
| 23 | Untreated immune serum + lysin. | Died after 8 hrs.; prostrated at once; hemoglobinuria. |
| 24 | Exhausted immune serum + lysin. | Remained well save for a moderate anemia. |

Guinea Pig 20 weighed 425 gm., the others 450 gm.
The order of injection was as follows: Nos. 24, 23, 21, 20, 22.

### *Comment.*

It is clear that by the method of selective absorption an antitoxic serum strong in tissue antibodies can be deprived of the latter to such extent as to be converted from a highly injurious agent into one capable of saving life. Indeed such a serum submitted to absorption five successive times, with a total bulk of corpuscles almost twice its own volume, was found to retain practically all its titer in antitoxin (Experiment 3).

A few of the guinea pigs receiving mixtures of exhausted serum and megatheriolysin were temporarily prostrated, lying on the side, and twitching, but they soon got to their feet and showed no permanent injury. The prostration occurred as frequently after the injection of normal serum as of that from animals immunized against guinea pig tissues. It is not possible to say whether the symptoms were due to "anaphylatoxin," or resulted from struggle under durance in connection with the rapid intravenous injection of a relatively large amount of foreign fluid. No such symptoms were ever observed after intraperitoneal inoculations.

The animals saved by the action of exhausted immune serum gave no evidence of injury to liver, spleen, or kidney, such as might perhaps have been expected in view of the fact that suspensions of these organs mixed with defibrinated blood were employed in the immunization. However, the only urine test made was for hemoglobin. Some of the guinea pigs receiving exhausted serum developed a slight or moderate anemia which was slow to appear and was most marked 4 or 5 days after the injection. Special experiments which need not here be cited in detail showed convincingly that the blood injury was due, not to unneutralized megatheriolysin, but solely to insufficient exhaustion of the serum with red cells, as proved by the persistence in it of hemagglutinins. When the absorptions were continued until all hemagglutinins had been removed, the serum was harmless to the blood. For example, it was found that the serum of Experiment 3 after seven absorptions no longer possessed hemagglutinins or produced anemia, whereas after only five absorptions it had both these characters as our protocol shows. The point is an important one, suggesting that the complete absence of hemagglutinins may be taken as the index to when exhaustion of a serum is complete. This indicator has been adopted, in much of our later work, and properly, as the results show. Hemagglutinins are far stronger than hemolysins in most sera resulting from prolonged immunization with animal tissues, and persist long after the latter have been removed by absorption.[7]

*Selective Absorption Applied to an Antipneumococcus Serum.*

The results of the work with an antitoxic serum were so encouraging that experiments were begun with sera of other types. It was highly desirable that they should be developed through the actual employment of infected tissues as antigen. The pneumococcus was selected for some of the tests, and attempts were made to immunize dogs against the organism by means of injections with the tissues of rabbits dying of pneumococcus septicemia. The difficulties encoun-

[7] An exception is to be noted in the case of anti-chicken sera from the goose and rabbit. These regularly contain hemolysins in fair quantity but only weak hemagglutinins.

tered illustrate strikingly the differences which may exist in the pathogenicity of a microorganism growing *in vivo* and *in vitro*.

Dogs possess a considerable resistance to the pneumococcus compared with some other species, as is well known; and they will often withstand the intravenous injection of several cubic centimeters of a bouillon culture fatal in minute quantity to mice. Intraperitoneal inoculations are even better borne. Nevertheless the immunization of dogs against the pneumococcus by intraperitoneal injections of blood or other tissues from rabbits moribund with pneumococcus septicemia proved well-nigh impossible, because of the high virulence of the antigen and our inability to standardize that derived from different rabbits. Small amounts of the infected tissue caused the dogs to die with a pneumococcus septicemia. Not infrequently they withstood a number of injections, only to succumb to one which was, quantitatively speaking, inconsiderable. In order to avoid this the infected rabbit tissue was heated *in vitro* at temperatures between 40° and 50°C. prior to injection, and the number of living organisms was thus reduced, as cultures showed, from millions to but a few per cubic centimeter. Still the injections often resulted fatally. Separate intraperitoneal inoculations of pneumococcus cultures and of normal tissues gave better results, but pneumococcus peritonitis so often ensued that at length separate subcutaneous inoculations were decided upon. These were carried out over a period of several months and a serum was finally obtained of sufficient antipneumococcus and anti-tissue titer to be suitable for experiments on selective absorption. We can confirm the observation of Nuttall[8] and Doerr and Moldovan[9] that antibodies of high titer are with difficulty elicited in the dog, as a response to immunization.

The plan of the experiments required yet a further alteration. Rabbits were found to vary so markedly in their resistance to the pneumococcus that very many would have been required had they been used as test animals in protection experiments with the antipneumococcus serum. It was decided, on this account, to exhaust the dog serum with rabbit red cells, in the manner that had proved successful with antimegatheriolytic serum and guinea pig cells, but to

[8] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

[9] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

carry out protection tests with mice instead of rabbits. Mice were found to tolerate well the intraperitoneal injection of normal dog serum in the amount necessary for the work.

*Immunization of Animals.*—Nine dogs weighing from 8¼ to 13 kilos were injected intravenously with amounts varying from 0.5 to 1.25 cc. of the mixed citrated blood of three rabbits moribund with pneumococcus septicemia. The organism was of Type I (Neufeld strain), and the infected blood was preserved in the frozen condition for 4 days prior to use. Five of the dogs died of pneumococcus septicemia within a few days after the injection. The surviving four animals received injections of antigen at intervals of 7 days for more than 2 months. At first citrated septicemic blood was given intraperitoneally. This was badly tolerated, so recourse was had to inoculations with normal tissue and bouillon cultures of the pneumococcus, given at separate subcutaneous sites and on different days. The normal tissue consisted of suspensions of finely ground rabbit liver, spleen, and kidney mixed with defibrinated blood. Kidney tissue in special was employed, with the object of obtaining a serum that would be nephrotoxic. None of the dog sera acquired a demonstrable nephrotoxin, however, though hemolysins and hemagglutinins soon developed, and also weak agglutinins for the pneumococcus. The two most highly immunized dogs (Dogs A and B), as judged by these features, were bled for serum 9 days after the last pneumococcus injection.

*Exhaustion of the Serum.*—The method of selective absorption was that already described in connection with antimegatheriolytic serum. Rabbit red cells, thrice washed, were packed by rapid centrifugation, all possible fluid was removed, and the cells were mixed and incubated with the serum under test. The latter was in this way exhausted by contact with several successive portions of cells.

*Experiment.*—The sera of two immunized dogs and three normal controls were inactivated at 56°C. for ½ hour, and portions of all were submitted to an exactly similar exhaustion with red corpuscles. The incubation period was 1 hour with each successive batch of cells.

| Mixture. | Hemagglutination. | | |
|---|---|---|---|
| | Immune sera. | | Normal sera. |
| | A | B | |
| 26 cc. of serum + 3⅓ cc. of red cells, incubated 1 hr. and | Massive. | Heavy. | |
| serum transferred to 3⅓ cc. of red cells; incubated 1 hr. | Moderate. | Moderate. | |
| " " " 4 " " " " " 1 " | Slight. | Slight. | 0 |
| " " " 4 " " " " " 1 " | Tr. | Ft. Tr. | |

Cultures taken after the last absorption showed all the sera to be sterile.

*Anti-Rabbit Titer of the Immune Sera. Hemolysis.*—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent red cells.

| Serum. | Serum dilution. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1,024 |
| Dog A Untreated.... | C. | C. | +++ | ++ | ++− | + | + | Tr. | Ft. Tr. | 0 | 0 |
| Dog A Exhausted.... | | | No hemolysis. | | | | | | | | |
| Dog B Untreated.... | C. (?) | Alm. C. | Alm. C. | ++++ | ++ | +− | +− | Tr. | Ft. Tr. | 0 | 0 |
| Dog B Exhausted.... | | | No hemolysis. | | | | | | | | |

*Hemagglutination.*—The mixtures were the same as those for hemolysis save that 0.2 cc. of salt solution was substituted for guinea pig complement.

| Serum. | Serum dilution. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1,024 |
| Dog A Untreated.. | C. | C. | C. | C. | +++ | +++ | ++ | Tr. | Tr. | 0 | 0 |
| Dog A Exhausted.. | ++++ | +++ | ++ | Tr. (?) | 0 | 0 | | | | | |
| Dog B Untreated.. | C. | C. | Alm. C. | ++++ | ++++ | ++ | Tr. | 0 | 0 | 0 | 0 |
| Dog B Exhausted.. | ++ | Tr. | 0 | 0 | | | | | | | |

Although some hemagglutination was noted in the mixtures with exhausted serum after they had stood over night, no trace of this was observable when they were first taken from the incubator. Many hemagglutinins, as Landsteiner and Reich[10] first showed, act most strongly at low temperature.

Because of these findings, which showed that the exhaustion of the immune sera with rabbit cells had been practically complete, no similar tests were made of the exhausted normal sera. For the anti-rabbit titer of these sera, when unexhausted, was, relatively speaking, slight.

*Pneumococcus Agglutinins.*—These were present in the immune sera but were weak, being effective on macroscopic test only in dilutions up to 1 in 16 of each serum. They were found to be unaffected by exhaustion of the sera. The details of the tests need not be given.

*In Vivo Tests of the Protective Power of the Exhausted Antipneumococcus Serum.*—With the change in plan that determined the use of mice instead of

[10] Landsteiner, K., and Reich, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 83.

rabbits for the protection experiments, it became unnecessary to make *in vivo* tests as to whether the sera exhausted with red cells had been deprived of their toxicity for the species furnishing the cells; that is to say, for rabbits. The results with exhausted anti-guinea pig serum of far higher original titer, which have already been described, were deemed sufficient on the point, especially since tests showed that the unexhausted dog serum contained not the least nephrotoxin for the rabbit, despite the repeated use of the renal tissue of rabbits as an antigen. The method adopted for the protection experiments with mice was that now familiar from the work of Avery, Chickering, Cole, and Dochez.[11] An 18 hour culture in pneumococcus broth of the Neufeld strain of pneumococcus was used in a series of tenfold dilutions with this broth. Of each culture dilution 0.5 cc. was drawn into a Record syringe, then 0.5 cc. of the serum under test was taken up, and the whole was at once injected into the peritoneal cavity of a 20 gm. mouse. The control sera were handled after the immune sera, so that the former had the advantage of any attenuation in the pneumococcus suspensions which might have occurred during the period of the injections. Last of all, some mice were given a set of control mixtures containing 0.5 cc. of broth instead of a serum.

The animals that died were autopsied promptly and films taken of the peritoneal exudate and heart's blood. These in every case showed the pneumococcus in pure culture. No mouse was put down as surviving until 4 days after injection.

| Serum. | | Amount of culture. | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.000001 cc. | 0.00001 cc. | 0.0001 cc. | 0.001 cc. | 0.01 cc. | 0.1 cc. |
| | | *hrs.* | *hrs.* | *hrs.* | *hrs.* | *hrs.* | *hrs.* |
| Broth controls | | D.* in 26 | D. in 28 | D. in 32 | D. in 20 | D. in 16 | |
| Immune A | Unexhausted | Lived. | Lived. | " " 42 | " " 26 | " " 23 | D. in 17 |
| | Exhausted | " | " | Lived. | " " 89 | Lived.† | " " 11 |
| Immune B | Unexhausted | " | " | " | Lived. | D. in 17 | " " 14 |
| | Exhausted | " | " | " | D. in 32 | " " 49 | Lived. |
| Normal a | Unexhausted | D. in 30 | D. in 30 | D. in 23 | " " 17 | " " 17 | D. in 7½ |
| | Exhausted | " " 31 | " " 21 | " " 20 | " " 16 | " " 11 | " " 9 |
| Normal b | Unexhausted | " " 27 | " " 30 | " " 25 | " " 22 | " " 12 | " " 9½ |
| | Exhausted | " " 31 | " " 50 | " " 22 | " " 11 | " " 9 | " " 9 |
| Normal c | Unexhausted | " " 30 | " " 42 | " " 19 | " " 14 | " " 14 | " " 7½ |
| | Exhausted | " " 40 | " " 28 | " " 28 | " " 22 | " " 12 | " " 10 |

* D. indicates died.

† Slight escape of injected fluid beneath skin.

[11] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

*Comment.*

The experiment shows that the immune dog sera protected mice against about 100 times the amount of pneumococci that was fatal when normal dog serum was employed. The exhaustion of the immune sera with four successive portions of rabbit red cells did not diminish in the least its protective character. The question may be asked, why was such an oblique method used to demonstrate the availability of the exhausted serum? Instead of immunizing dogs with rabbit tissue, exhausting the dog serum with rabbit red cells, and testing protection on mice, might not these latter animals have been employed throughout, to the elimination of rabbits? This was not practicable for several reasons. It would have been difficult to obtain enough sterile normal tissue from mice for the production of a strong anti-mouse serum, and, granting that such a serum could eventually have been elicited, the problem would have arisen of obtaining sufficient mouse red cells for its exhaustion. Undoubtedly the spleens of mice dying of pneumococcus infection would have furnished a powerful antigen, as concerns this organism; but so little of the splenic tissue could have been employed in the immunization, owing to the virulence of the pneumococci therein contained, that it is doubtful whether the serum resulting would have been strongly anti-mouse. And a serum strong in anti-tissue elements was desirable for our type experiments.

*Selective Absorption Applied to a Serum Conferring Protection against Poliomyelitis.*

As enlarging the general scope of the work, a test was made of whether exhaustion with red cells would deprive the serum of a monkey recovered from poliomyelitis of its protective power against this disease.[12]

The serum of an immune monkey might conceivably be used in the treatment of human beings after exhaustion of its anti-human elements. For this reason the selective absorption was carried out with human cells, though in the ultimate test of protection monkeys were of

[12] The experiment was rendered possible through the cooperation of Dr. Amoss.

necessity employed. The choice of cells was a poor one, because the anti-human elements in monkey serum are extremely weak[13] and are not readily enhanced by tissue injections. The test of the persistence of antipoliomyelitic elements in serum submitted to selective absorption with human cells is in consequence not a drastic one.

*Experiment.*—A *Macacus rhesus* monkey recently recovered from poliomyelitis, and with severe residual paralyses, was given intravenously on 3 successive days portions of a mixture of defibrinated human blood and an extract in salt solution of human placenta ground with sand. 10 days after the last injection the animal was bled for serum. This on test showed no hemolysin for human red cells and only weak agglutinins. It was exhausted as follows, according to the usual technique.

| Mixture. | Hemagglutination. |
|---|---|
| 2.25 cc. of serum + 0.5 cc. of human red cells, incubated 5 min.; | Massive. |
| and serum transferred to 0.25 cc. of human red cells, incubated 45 min.; | Moderate. |
| and serum transferred to 0.25 cc. of human red cells, incubated 45 min. | Faint. |

The exhaustion was nearly complete, as shown by tests in which one part of serum in graded dilutions + one part of 5 per cent human red blood cells were mixed in Wright's tubes and examined microscopically after 15 minutes at room temperature.

*Agglutination.*

| Serum. | Serum dilution. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |
| Untreated | C. | − | − | + | +− | Ft. Tr. | 0 |
| Exhausted | + | Tr. | | | | | |

The test of protective power was carried out by Dr. Amoss, who mixed 2 cc. of the exhausted serum with 0.2 cc. of freshly prepared poliomyelitic virus, and injected the whole, after 2 hours incubation, into the cerebrum of a normal *rhesus* monkey. The animal was one of a considerable number receiving an equal amount of the same virus mixed with various sera, so the experiment was well controlled. Eight monkeys were given mixtures of 0.2 cc. of virus + 2 cc. of

[13] Marshall, H. T., *J. Exp. Med.*, 1901–05, vi, 347.

normal human or monkey serum, and all came down with poliomyelitis after from 5 to 7 days and died. The animal receiving exhausted immune serum mixed with virus remained entirely free from the disease.

The indication from this one experiment is clear, that the principle neutralizing the virus of poliomyelitis persists in immune serum exhausted with red cells. Owing to the difficulty of obtaining immune monkeys the work has not been repeated.

## SUMMARY.

Attempts to produce antisera in animals to combat specific infections are usually deferred until the cause of the infection has been isolated and grown in pure culture to furnish antigen. It has seemed to us that the fulfillment of these conditions might in some cases be rendered unnecessary through the use of infected tissue itself as an antigen, combined with selective absorption of the antiserum to rid it of elements injurious to the species furnishing the tissue. In order to test this possibility type experiments have been carried out with immune sera effective against known antigens of three different sorts:

1. Sera resulting from the injection of rabbits and a goat with normal guinea pig tissues and a bacterial hemotoxin, the megatheriolysin described by Todd, which hemolyzes guinea pig cells. The sera possessed strong antitoxins for the megatheriolysin but were fatal to guinea pigs. By the method of selective absorption they were rendered innocuous to these animals and were successfully used to protect them from lethal doses of the megatheriolysin.

2. Anti-rabbit dog sera containing antibodies protective against pneumococcus infection. Such sera, subjected to repeated absorption with rabbit red cells, proved capable of protecting mice from pneumococcus infection in exactly the same degree as the unexhausted serum; that is to say, they protected against 100 times the dose of pneumococci that was fatal with normal dog serum.

3. The serum of a monkey recovered from poliomyelitis and repeatedly injected with human red cells and extract of placental tissue. This serum, after selective absorption with human red cells, protected a monkey against an intracerebral dose of poliomyelitic virus

fatal to eight other monkeys given it with normal monkey or human serum.

The results in these instances, purposely chosen for their simplicity, would seem to indicate for the absorption method some usefulness in the study of immunity to infections of unknown cause. In Part II of our paper the method is applied to one such infection; namely, a sarcoma of the fowl engendered by a filterable agent. A general discussion will be found in connection with this portion of the work.