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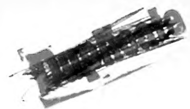

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# ON LOCAL AND GENERAL IMMUNITY[1]

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There is considerable evidence that natural resistance, and whatever grade of active acquired immunity may exist against streptococcus infections, is due to a mechanism that differs from that which is operative in many other bacterial infections. It seems likely that ignorance of what constitutes this difference accounts to a large extent for the failure to obtain any strikingly successful results in the prevention or cure of any of the manifold forms of streptococcic disease.

There seems to us an increasingly generalized acceptance of the main points of Metchnikoff's phagocytic theory of immunity, at least in the following features: the majority of bacteria that invade the animal body are dealt with first by the mobile and ubiquitous polymorphonuclear leucocytes. These phagocytes either act alone or in conjunction, particularly in conditions of acquired immunity, with the tropinizing substances present in serum. There seem, however, to be instances of immunity in which neither of these two factors seem particularly operative. Natural resistance as represented by phagocytes is not sufficient alone to protect the body, let us say, against normally virulent typhoid bacilli; phagocytes have no increased destructive power for the bacillus after recovery from typhoid fever, antibodies soon disappear, and yet a most marked resistance to typhoid infection is present. We have been accustomed to explain an instance such as this as due to tissue immunity.

There would seem to be no consensus of opinion as to how either the normal or the immunized animal disposes of streptococci.

[1] Presidential address before the American Association of Immunologists, Washington, May 1, 1922.

The problems may or may not be identical, but we do at least know this, that normal animals are able to dispose of a certain number of avirulent organisms, and actively immunized animals or animals that have received immune serum are able to dispose of even virulent streptococci. The early investigations in this field would attribute the predominant importance to the phagocytes. Metchnikoff in his original confirmation of Fehleisen's work on the production of experimental erysipelas attributed the disposal of the cocci in the lesion to the intervention of polymorphonuclear leucocytes, although he here for the first time brings out the importance of the mononuclear cells or "macrophages" in disposing eventually of the polymorphonuclears that have ingested the bacteria. The subsequent work of Bordet, Tchistowitch and others would likewise lay emphasis on the phagocytes, both in active and passive streptococcus immunity. Reudiger has apparently demonstrated that leucocytes and bone marrow cells destroy a certain number of avirulent streptococci, whereas other body cells do not. Denys and LeClef attribute protection against recurrent erysipelas in the rabbit ear to a more successful degree of phagocytosis by polymorphonuclears.

Another group of more recent articles would tend to minimize the importance of microphages in the destruction of streptococcus. Baumgarten makes the definite statement that polymorphonuclears have no function in disposing of these microörganisms, and regards antibodies alone as operative in erysipelas. He does not, however, give any particulars as to the mode of action of these suppositious bodies, which indeed under the conditions he describes are not likely to be at hand. We have already referred to the observations beginning with Metchnikoff and reiterated more concretely by MacCallum that the mononuclear cells are predominant in lesions of erysipelas. Zangemeister and Gans have called attention to the important prognostic significance of the mononuclear response when streptococci of varying pathogenicity are injected by various routes into monkeys. The accumulation of cells of the macrophage type is likewise indicated by their occurrence characteristically in streptococcus myositis (Rosenow and Ashby) and similar cells more clearly described

by Cecil in myocardial and in joint infections which develop apparently from the vascular endothelium.

There would seem, then, to be a gradually increasing conjecture—it can scarcely be called more—that macrophages, which we interpret to mean primarily cells of fixed tissue and often local origin, are in some way connected with prolonged and sometimes healing processes in streptococcus infection. These indications of macrophage intervention do not rule out such observations as those of Weil, who by a process of exclusion comes to the conclusion that the fixed tissue cells rather than any material present in the blood serum is responsible for streptococcus resistance. The more concrete and recent observations of Hopkins and Parker show that when streptococci are injected into the circulation of cats they are found more numerously in the lung, where they are killed in from five to eight hours. This local bactericidal property would seem to be dependent on the living cells and not on their extracts. Similar results have been obtained by Bartlett and Osaki with staphylococci. The early observations of Wyssokowitsch also pointed to the importance of phagocytosis by endothelial cells. This last observation is further corroborated by Hopkins and Parker. No very certain explanation as to whether the increased resistance of immunized animals is due to the same mechanism as this one described by Hopkins and Parker is available.

The most recent and significant observation on streptococcus immunity has, we believe, been made by Levaditi, who found that war wounds which are infected with streptococcus tend to clear up at irregular intervals. In a wound from which the microörganisms have disappeared there is found the property of destroying rapidly considerable numbers of the streptococci that had previously infected it. When the infected bandages from another wound on the same individual that has not become sterile are applied to the wound that has become sterile, the microörganisms rapidly disappear. If, however, cultures of streptococcus from another source are applied they do not disappear so rapidly. This, in our opinion, points not simply to a specific destruction of bacteria by the cells present in granulating wounds, but to a

localized function present in one wound and not in another in the same body. This suggestion reaches back into the more general question of the actual existence of a local as compared with a general immunity.

In discussing the question of proof for or against existence of a strictly local, as contrasted with a general, form of immunity, it would seem that in this connection no sufficiently careful separation has been made between the two main types of immunity; namely, active and passive. The form of local protection, which has been so aptly termed by Wassermann and Citron, "Umstimmung" or "retuning" of the tissues, so that they react in a new fashion, is what we understand by local immunity. In other words, it is an active process on the part of individual groups of cells, and our interest in this connection lies in an attempt to determine whether this "Umstimmung" may exist in one part of the body as against other parts. When we analyze the work that has been done in an attempt to prove the existence of local immunity, we agree with Hektoen that most of it, including the very work of Wassermann and Citron, on which this "retuning" conception was based experimentally, is not strictly demonstrative. The work of Roemer on local corneal immunity to abrin, of Von Dungern on crab plasma, of Miyashita on the corneal production of hemolysins and particularly the experiments of Wassermann and Citron, do nothing but evidence the localization in certain areas of antibodies from the general circulation, which is indicative, in other words, of a local mobilization of a general form of protection. In addition to this interpretation of their results, strong criticism on the experimental methods involved have been made by Hektoen, with whom we heartily agree. In a similar manner the active immunization of children against diphtheria toxin by local application to the nasal mucosa by Dserzgowsky and by Blumenau are further instances of the same mechanism. Hektoen was further unable to prove the local formation of hemolysins and Mutermilch was equally convinced that there is no local formation of antibodies to trypanosomes after intrapleural inoculation.

A very early and real indication of what we understand by

local immunity was apparently obtained by Loeffler in 1881. To quote from Miyashita, Loeffler found that the inoculation of the organism of mouse septicemia in the rabbit produces a fatal infection on subcutaneous inoculation and a local keratitis on corneal inoculation. It would seem, however, according to Loeffler, that if the animal is inoculated on the right ear and then eight days later on the left, no reaction occurs. The cornea, however, is not protected until the third week, which coincides with appearance of generalized immunity. It would seem here then that we have an area of rather localized protection occurring before general protection, and subsequently the protection of more remote localized areas, is assured. The remarks of Koch in criticism of Pasteur's method of anthrax immunization would seem to appear significant in this connection. Koch found that although sheep immunized against anthrax by subcutaneous inoculation are protected against artificial subcutaneous inoculation of anthrax, they are less thoroughly protected against ingestion of anthrax spores, which is at least one of the ordinary methods of natural infection.

Besredka (1, 2, 3, 4) has recently reported some most interesting studies, which bear directly on this question. His observations concern first of all infections with typhoid and with paratyphoid $\beta$ organisms in rabbits (2). He found that although rabbits can not normally be infected by ingestion of typhoid bacilli and at least of certain varieties of paratyphoid $\beta$, they can be rendered more susceptible by previous ingestion of ox bile, so that they die within a few days with intestinal lesions that are said to resemble those in human typhoid fever. So far the observations have been confirmed by Zingher and Soletsky. Besredka's further observations are of even greater importance; they have, we believe, received no confirmation, and are denied by the last quoted investigators. The author claims that although living paratyphoid $\beta$ organisms given per os produce no infection or immunity in rabbits if he treats the cultures with bile or gives an animal bile before the cultures by the mouth, agglutinins are formed and the animal is protected against intestinal infection in the manner above indicated. In subsequent papers Besredka

has apparently shown that organisms that are very pathogenic for rabbits by the intestine or intravenously, such as paratyphoid $\beta$ and *B. abortus*, may be given in larger doses by the trachea (3). The susceptibility even by this route is lowered by the previous inoculation of bile and recovery from an original injection of the cultures is followed by an increased general resistance of the animal even to intravenous infections and is accompanied by the presence of antibodies in the circulation. In these papers he further suggests the possibility that the local resistance in the trachea has been increased, although the experiments themselves do not definitely prove it. In still further continuation of this work Besredka (4) has shown that infection of guinea pigs by means of *B. anthracis* can be prevented most efficiently by immunizing the skin rather than the body as a whole. Intracutaneous injection or rubbing the cultures into a shaved skin produces a local lesion, but not a general infection, as is produced by subcutaneous inoculation. The animals are, however, subsequently well protected against infection by any route. Besredka regards this as a form of strictly local immunization. Protection of the skin results, according to him, in closing the normal portal of entry of the anthrax bacillus. Indeed he would regard entrance through this portal as essential in producing infection. He makes the surprising statement that a general septicemia can not be produced by local inoculation into the peritoneum or circulation unless the infection first passes through the skin. Skin immunity then, to judge from his explanations at least, if not from his experiments, is essential in immunizing against anthrax. It may be produced as a sequel to a condition of general immunity, but is more readily and less harmfully effected by inoculation into the superficial layers of the skin. These observations recall again the statement of Koch in criticism of Pasteur's immunization against anthrax in sheep, to which a reference has been made.

The whole question of the local reactions of so-called hypersusceptibility which occur in immunized and infected animals passes through the mind at this point. Certain of these tests, such as the typhoidin reaction, the Jenner reaction of re-vaccination and

perhaps the tuberculin reaction, I have already discussed in their prognostic sense (Gay), as indicative of protection rather than as simple diagnostic signs. Levaditi (2) has recently shown that the intensity of the local reaction to killed streptococci in patients recovering from streptococcic wounds varies directly with their ability to heal them. Are these local reactions true instances of anaphylaxis (Calmette)? Is local hypersusceptibility different from general hypersusceptibility in the sense of Zinsser? And furthermore, what is the relation of anaphylaxis, either general or local, to general or local immunity?—But you see where such a discussion will lead us, and we had better restrict ourselves to a more limited field.

We have, we believe, found the method at least by which the question of the existence of local immunity may be settled. I think, moreover, that we have proceeded to a measurable degree in proving that local immunity does exist in streptococcus infections. For some years we have been working with the local lesions in rabbits produced by injection of a strain of *Streptococcus pyogenes* originally isolated from a case of human empyema. This strain of streptococcus, as isolated and as preserved by frequent transfer on a blood agar medium, has little pathogenic properties for rabbits. It does, however, in large doses and somewhat irregularly, produce a purulent exudate when injected directly into the rabbit pleura. By means of frequent passage through the pleural cavity, the organism became enhanced in virulence, so that a very small amount (0.001 cc.) of this passage pleural culture will regularly produce a fatal empyema. The virulence, moreover, may be maintained at this point by conservation of the culture in the pleural fluid. This passage pleural culture not only produces pleurisy regularly, but kills rabbits of general septicemia, when given intravenously, and produces either death or a recoverable form of erysipelas when injected intradermally. In other words, the general pathogenicity is increased without a localized selective affinity. The fatal dose by the pleura, both with the original stock culture and the passage culture, is much less than by the other routes mentioned.

In recent months the study of the experimental erysipelas pro-

duced by the passage culture, but not by the stock culture, has interested us exceedingly. Experimental erysipelas was first studied with pure cultures of streptococcus by Fehleisen and subsequently by a number of observers, notably Roger, Denys and Leclef, Cobbett and Melsome. It was also shown by some of these investigators, beginning with the work of Meierowitsch, that some form of immunity against erysipelas follows recovery from this experimental infection. The exact conditions surrounding this resistance have never been clearly worked out and indeed no apparent interest has been taken in this experimental lesion for many years. With the passage culture of fixed virulence we can produce erysipelas regularly with a dose of 0.1 cc. of a twenty-four-hour broth culture. The animal recovers perfectly, although twice this dose leads to a fatal septicemia. Recovery from erysipelas protects an animal completely against re-inoculation intradermally elsewhere on the body. It does not, however, protect the animal against intravenous inoculation with the same dose, and it should further be mentioned that the minimal lethal dose is practically the same intravenously as is the symptomatic dose intradermally. The converse of this is also true. Intravenous inoculation of sublethal doses protects the animal against intravenous inoculation, but not against intradermal inoculation.

A similar set of conditions is strongly indicated from our attempts to produce protection against intrapleural inoculation, where, however, the proof is not so definite as yet, owing to the extreme susceptibility of rabbits when inoculated by the intrapleural route. We have hitherto in most experiments used too many multiples of the lethal dose to demonstrate the desired effect. But in several experiments at least it has been possible to show that a given harmless dose of the stock culture, when administered intrapleurally for immunization purposes, will protect against intrapleural infection, whereas the same dose given intravenously will not protect; and the reverse of this effect is also apparent. Intravenous immunization protects against intravenous infection, but the same amount of immunization intrapleurally does not.

Experiments along this line will, we believe, lead to the dem-

onstration of true local immunity, which, we believe, from the references that we have quoted is of considerable importance in streptococcus infections. It appears evident, moreover, that an appreciation of the local nature of some forms of acquired resistance to bacteria may eventually be of service in improving our method of combating localized infections.

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# HEREDITARY BLOOD QUALITIES: STATISTICAL CONSIDERATIONS

REUBEN OTTENBERG

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In the original publication of von Dungern and Hirschfeld, and in my recent articles on the subject, there is sufficient evidence to prove the Mendelian nature of the inheritance of the human blood groups and to demonstrate the mechanism as depending on the dominance of agglutinogen B over absent B and of agglutinogen A over absent A.

For this reason, and in order to make my former presentations as simple as possible, I there omitted the comparison of the percentages of different groups actually found with the percentages to be expected from the mendelian law. However, as this comparison, though not essential, is of some value as additional evidence, and as so little is yet known about the inheritance of any normal human characters, I wish to set forth this comparison with regard to the 255 persons presented in my article.

In order to avoid repetition I will refer the reader to my last paper (1) for the facts on which the present article is based. Instead of the terminology A, not A, B, not B, used in my former publications (2), I will use the terms A, a, B, b, for the dominant and recessive qualities, since the latter terminology is simpler and is in general use by students of genetics.

The study of human heredity is beset with certain difficulties which do not occur in studying the heredity of the lower animals and the plants.

If we could start with equal numbers of pure AA and pure aa individuals the numbers of AA, Aa and aa to be expected would follow the mendelian formula 1:2:1. If we could start with any known numbers of AA and aa persons it would be easy to calcu-

late by cross multiplication of germ cell ratios the proportions to be expected.

But though we can count the number of persons who show A and who show a, we do not know the proportion of the respective kinds of germ cells because we do not know how many of the persons showing A are pure AA and how many are hybrid Aa.

This can be determined approximately, however, according to the very ingenious method adopted in von Dungern and Hirschfeld's paper (3), by calculating backward and finding what proportion of pure AA, hybrid Aa, and pure aa, in preceding generations would give the proportion of A and a found in the present population.

This was done by von Dungern and Hirschfeld for their statistical material in an empirical manner, by trying at random various proportions of AA, Aa, and aa, and multiplying out the expected progeny until they found such a proportion as would keep the actual ratio of apparent A and a individuals produced approximately permanent in successive generations and equal to that actually found. Thus A and a were about equal in numbers in their material; they found that a ratio of 1AA:4Aa:5aa would result in 9AA:42Aa:49aa in the next generation, and in the next, 3,600AA:168,000Aa:196,000aa; i.e., about the same proportion in each generation. (These figures they arrived at by computing the ratios of the total number of A germ cells and of *a* germ cells. Thus with 1AA:4Aa:5aa, there will be 6A to 14a germ cells, and the offspring will (see diagram) be 36AA:168Aa:196aa (a ratio of 9:42:49 or nearly 1:4:5.)

| | | *Sperm* | |
|---|---|---|---|
| | | 6 | 14 |
| | | A | a |
| *Ova* | 6 A | 36 AA | 84 Aa |
| | 14 a | 84 Aa | 196 aa |

This empirical method of trial and error is laborious and the same result can more quickly be reached by a simple algebraic calculation. As this may be of use in future to other persons who wish to determine the agreement of their actual with their

theoretical findings I present the calculation here as applied to my material. (The proportions of the groups in von Dungern and Hirschfeld's material is quite different from that in my material.) My cases show: group I, 111 persons; group II, 87 persons; group III, 35 persons; group IV; 12 persons.

That is, there are apparent 99A persons (group II and IV) to 146a (not A) persons (groups I and III) a ratio of approximately

$$A : a :: 2 : 3.$$

The problem is to find out what proportion of individuals of constitution AA, and what of constitution Aa, there must be among the A persons in order that the succeeding generations will always show the same ratio

$$A : a :: 2 : 3.$$

If we call the ratio of AA to Aa, x:y, then the proportion of aa occurring will be $\frac{3}{2}(x+y)$, (since the total number of a individuals is $\frac{3}{2}$ of all the A individuals); i.e.,

$$x : y : \tfrac{3}{2}(x + y) :: AA : Aa : aa.$$

From this, the relative number of germ cells A and a produced by this generation will evidently be

$$A = (2x + y)$$
$$a = 2 \times \tfrac{3}{2}(x + y) + y = (3x + 4y)$$

If we multiply these out as indicated in the diagram

| | | | *Sperm* (2x + y) | (3x + 4y) |
|---|---|---|---|---|
| | | | A | a |
| *Ova* | (2x + y) | A | AA | Aa |
| | (3x + 4y) | a | Aa | aa |

we find that the resulting offspring are:

$$AA,\quad (2x + y)^2$$
$$Aa,\quad 2(2x + y)\ (3x + 4y)$$
$$aa,\quad (3x + 4y)^2$$

Since the ratios of AA:Aa:aa in the two generations are, according to supposition, equal, we can set them so:

(I) $x : y : \frac{3}{2}(x + y) :: (2x + y)^2 : 2(2x + y)(3x + 4y) : (3x + 4y)^2$

We can easily solve this for x in terms of y. Thus using the last two terms on each side of proportion I

$$\frac{y}{\frac{3}{2}(x + y)} = \frac{2(2x + y)(3x + 4y)^2}{(3x + 4y)^2}$$

which simplifies to

$$\text{(II)} \qquad \frac{y}{3(x + y)} = \frac{(2x + y)}{(3x + 4y)}$$

Multiplying out and solving for y in terms of x we get

$$y = x \times 6.87 \text{ (approximately)}$$

Therefore

$$AA : Aa : aa :: 1 : 6.87 : 11.80$$

$$\text{(or roughly } 1 : 7 : 12)$$

From this we can now calculate the proportion of group I children to be expected from marriages of two group II persons by the method used by von Dungern and Hirschfeld.

According to the mendelian principle the recessive quality, a, can only be apparent in the offspring when it is present in both parents, and then it will appear in $\frac{1}{4}$ of the offspring.

The chance of one group II (i.e., A) parent being in constitution Aa is according to the above, $\frac{687}{787}$ and the chances of both parents being so are therefore $\frac{687}{787} \times \frac{687}{787}$. So that the chances for group I (i.e., a) children are

$$\frac{687}{787} \times \frac{687}{787} \times \frac{1}{4} = 19 \text{ per cent (approximately)}$$

That is from A-A marriages (in my statistical population) the expectation of *a* (group I) children is 19 per cent and of A (group II) children 81 per cent. There were actually found 4a and 19A, or 17.4 per cent a and 82.6 per cent A.

For marriages of groups I and II the proportion of I and II (i.e., of a and A) children can be calculated as follows:

Of all group II persons (in my population), $\frac{687}{787}$ = 87.3 per cent are hybrid (i.e., Aa in composition) while $\frac{100}{787}$ = 12.7 per cent are pure AA.

Since all the children of the pure AA-aa marriages must show the quality A, 12.7 per cent of all children from I-II marriages must show A. In addition since Aa compose 87.3 per cent of the group II population and according to the mendelian principle the children of Aa-aa unions will show A in half the cases, a in the other half, one can expect $\frac{87.3}{2}$ per cent = 43.6 per cent A (group II) from this cause.

Or, the total of group II (A) children to be expected from I-II unions are 12.7 + 43.6 = 56 per cent, and of group I (a) children 44 per cent.

There were actually found 24A and 28a, or 46 per cent and 54 per cent, respectively.

In a similar way the calculation is carried out for the marriages of persons having B (chiefly group III). In my material there is a total of 47 B (groups III and IV) persons as against 198b (not B, i.e., groups I and II) persons or B:b::1:4 (roughly).

To find out what proportion of the B (group III) individuals must be BB and what Bb, if succeeding generations are to show B:b::1:4, let us set BB = q, Bb = r.

(III) Then BB : Bb : bb :: q : r : 4 (q + r)

From this the proportion of B germ cells will be (2q + r) and of b germ cells will be (8q + 9r). Calculating from this ratio of germ cells

| | | | *Sperm* | |
|---|---|---|---|---|
| | | | (2q + r) | (8q + 9r) |
| | | | B | b |
| *Ova* | (2q + r) | B | BB | Bb |
| | (8q + 9r) | b | Bb | bb |

The proportion of individuals in the next generation will be:

(IV) BB : Bb : bb :: $(2q + r)^2 : 2(2q + r)(8q + 9r) : (8q + 9r)^2$

Since (III) and (IV) are by supposition equal,

$$\frac{q}{r} = \frac{(2q + r)^2}{2(2q + r)\ (8q + 9r)}$$

which simplifies into $16(q^2 + qr) = r^2$. Solving for q,

$$q = r\ \frac{(\sqrt{5} - 2)}{4} + 4) = r \times 0.06 \text{ (approximately)}$$

Or substituting in (III) the values 6 and 100 for q and r

BB : Bb : bb :: 6 : 100 : 424, (or roughly 1 : 16 : 70)

Reasoning as before the recessive b can only appear as the offspring of III-III unions when both parents carry it (i.e., are Bb), and then it will appear in $\frac{1}{4}$ of the offspring. The chance for one parent being Bb is $\frac{100}{106}$ and for both being so is $\frac{100}{106} \times \frac{100}{106}$. Therefore the expectiation of b (group I) offspring from B-B (III-III) unions is

$$\frac{100}{106} \times \frac{100}{106} \times \frac{1}{4} = \frac{10000}{44944} = 22 \text{ per cent (approximately)}$$

and the expectation of B (group III) offspring conversely 78 per cent. There were actually 2b and 8B, or 20 per cent and 80 per cent respectively.

A similar kind of reasoning gives us the proportion of B and of b children to expect from group I-III marriages.

Of group III (or substance B) persons in my series $\frac{6}{106} = 5.6$ per cent (approximately) are pure BB, 94.4 per cent are Bb.

Of the 5.6 per cent BB, all the unions with group I (i.e. of BB-bb unions) will result in group III children only.

Of the 94.4 per cent Bb, in union with group I (i.e. of Bb-bb unions) the children will be half B, half b. Hence the expectation of B (group III) children from I-III marriages will be $5.6 + \frac{94.4}{2} =$ 52.8 per cent and conversely of b (group I) 47.2 per cent. There were actually 23B, 19b, or 54.7 per cent and 45.3 per cent respectively.

If we make a table of these calculated results and compare

them with the results actually found we see as good agreement as can be expected in so small a series.

| PARENTS | CHILDREN | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Per cent expected | | | | Per cent found | | | | Numbers found | | | |
| | A | a | B | b | A | a | B | b | A | a | B | b |
| AA (II-II and II-IV) ...... | 81 | 19 | | | 82.6 | 17.4 | | | 19 | 4 | | |
| Aa (II-I and II-III) ....... | 56 | 44 | | | 46.0 | 54.0 | | | 24 | 28 | | |
| aa (I-I and I-III).......... | 0 | 100 | | | 0 | 100.0 | | | 0 | 53 | | |
| BB (III-III and III-IV) .. | | | 78.0 | 22.0 | | | 80.0 | 20.0 | | | 8 | 2 |
| Bb (III-I and III-II).... | | | 52.8 | 47.2 | | | 54.7 | 46.3 | | | 23 | 19 |
| bb (I-I and I-II).......... | | | 0 | 100.0 | | | 0 | 100.0 | | | 0 | 80 |

The very close percentage agreements in the cases of A-A parents, of B-B parents and of Bb parents in view of the small actual numbers, can only be regarded as lucky accidents. The figures for A-a marriages do not show quite as close a correspondence between expected and actual percentage as do those of von Dungern and Hirschfeld. The complete agreement of the a-a and b-b figures of course is the most essential point in confirmation of the dominance of A and B.

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# THE RELATION OF ANTIGEN TO ANTIBODY (PRECIPITIN) IN VITRO

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There is little accurate knowledge concerning the fate of foreign protein introduced into the body. It appears in the blood and in some instances is excreted in relatively small quantity in the urine but this method of elimination offers no satisfactory explanation of its long continued persistence in the blood nor of its final disappearance. Those who have studied the fate of protein introduced into immunized animals have found that the antigen enters the blood and some have observed no difference in rapidity of disappearance between the normal and the immune animal. The subject is one of broad interest for there is no line of separation between relatively bland substances such as horse serum or egg white on the one hand, and on the other hand toxic substances such as the serum of lower vertebrate and of invertebrate species and other products of the animal or vegetable kingdom.

The subject is further obscured by the uncertainty of present knowledge concerning the action of the precipitin of the immune animal upon its antigen when the two meet within the living body. Appropriate reactions indicate that precipitin and the antigen used to produce it may be present in the same serum though they fail to unite to form precipitate. During certain phases of immunization antigen injected into an animal makes its appearance in the serum and is demonstrable even though the serum possesses a high precipitin titre. Antigen and antibody appear to exist side by side in the clear serum and do not unite to form a precipitate. Some of those who have studied this phenomenon have come to the conclusion that antigen and anti-

body (precipitinogin and precipitin) do not unite in the living body to form precipitate. It has been the purpose of the present study to determine if the interaction of antigen and antibody observed in vitro occurs within the body and to determine if precipitation has a part in the fixation or elimination of foreign protein.

In the supernatant fluid over a precipitate formed in a mixture of horse serum and serum of a rabbit immunized against horse serum Linossier and Lemoine (1) and Eisenberg (2) found both antigen and antibody demonstrable on the one hand by addition of antiserum and on the other by addition of antigen. In every precipitin reaction after an equilibrium has been established between the two components of the system, antigen and antibody, according to Eisenberg, coexist in the fluid without combining and only by addition of fresh increment of either reagent can this equilibrium be disturbed.

Other observers employing similar material have obtained identical results but von Dungern (3) using the serum of rabbits immunized against the blood plasma of crustacea and of mollusks found that both antigen and antibody could seldom be demonstrated in the supernatant fluid above a precipitate provided tests were made with the same antigen and antiserum used to produce the original reaction. V. Dungern has maintained the view that horse serum contains a variety of substances which act as antigens and produce corresponding antibodies. These antibodies are present in varying concentration in the serum of the immune animal and when horse serum is added to the antiserum one antibody may be completely fixed by its antigen whereas another antibody may be present in excess so that it remains uncombined. Similarly antigen may be present in excess of its antibody. The antigens and antibodies which coexist in the serum are not those which react with one another. Nevertheless a precipitin will be formed by the addition of horse serum containing all of the substances which have acted as antigens and furthermore the addition of antiserum containing all of the antibodies which have been formed in response to the injection of horse serum will cause a precipitate.

When egg albumin and egg globulin were separated by chemical means and used to immunize rabbits Weil and Coca (4) found that each of the resulting antisera acted strongly with its own antigen but very weakly with the other. Wells (5) has shown by means of anaphylactic reactions that five different antigens separable by chemical methods as ovomucoid, ovovitellin, albumin and two globulins can be obtained from hen's eggs.

In the attempt to obtain an antiserum containing a single antibody purified crystalline egg albumin has been used as antigen. Weil(6), who like previous observers had found that the supernatant fluid over a precipitate formed by horse serum and anti-horse serum from rabbit contained both antigen and antibody, obtained entirely different results when crystalline egg albumin was employed. If this substance is mixed in varying proportions with the serum of a rabbit immunized against it and the resulting precipitates removed by centrifugalization, the supernatant fluid may contain either antigen or antibody but never contains both. He has reached the conclusion that antigen and precipitating antibody do not coexist in the same fluid without undergoing union and precipitation. In accord with von Dungern he has believed that a multiplicity of antigens and of antibodies in horse serum explains the presence of both antibodies and antigens in precipitating mixtures.

The observations of Weil are not confirmed by Bayne-Jones (7) who has employed as antigens pure edestin prepared from hemp seed and crystalline egg albumin. When the pure protein antigen has been mixed in different proportions with serum of a rabbit immunized against the same substance and the resulting precipitate removed by centrifugalization, phases in the reaction have been found in which the antigen and its precipitin have been coexistent but ununited in the supernatant fluid.

The presence of both antigen and antibody in the serum of an immunized animal according to Zinsser and Young (8) cannot be explained by the laws of mass action applicable to the chemical change which occurs in a solution containing two dissociable substances for according to observations of Gay and Rusk, (9) confirmed by Zinsser and Young, the serum does not fix comple-

ment. Were the conception of mass action applicable to the reaction which occurs between precipitinogen and precipitin each of the two reacting bodies would be free and dissociated in the supernatant fluid and there would be a definite quantity of the united complex after equilibrium had been established. Zinsser and Young regard von Dungern's complex conception of a multiplicity of antigens and antibodies as unnceessary and find an analogy for the phenomenon in the reaction which occurs between a colloidal suspension of arsenic trisulphide and gelatin in the presence of gum arabic which acts as an inhibiting or protective agent, preventing precipitation of arsenic trisulphide.

## INTERACTION OF ANTIGEN AND PRECIPITIN IN VITRO; INHIBITION OF PRECIPITATION BY EXCESS OF ANTIGEN

In order to determine the strength of an agglutinating serum a definite quantity of bacterial suspension is brought into contact with diminishing quantities of serum and the titre of the serum is the highest dilution which causes demonstrable agglutination. The method has not been found convenient for the measurement of the strength of specific precipitating sera so that it is the prevailing custom to estimate the activity of these sera by mixing a definite quantity of the immune serum with varying dilutions of the antigen used in its production. The strength of the serum is indicated by the highest dilution of antigen which produces a visible precipitate when mixed with an amount of serum representing a large fraction (usually a fifth) of the volume of diluted antigen.

In the attempt to obtain a clearer understanding of the relation of antigen to precipitating antibody increasing dilutions of immune serum have been mixed with increasing dilutions of antibody. In the experiment recorded below equal quantities (0.5 cc.) of different dilutions of horse serum in normal salt solutions, and of the serum of a rabbit (no. 214) which had been repeatedly injected with horse serum, were brought together; the dilutions present after mixture are indicated in the table and the relative amount of precipitate is shown by plus signs.

In this and subsequent tables zero (0) means no precipitate; a dash (–) indicates that no test was made. In this experiment dilutions of antihorse serum above 100 were tested after the results with dilutions below 100 had been obtained.

A maximum amount of precipitate has been obtained by bringing together immune serum diluted 1:5 with antigen diluted 1:1000; an increase or decrease of the quantity of antigen decreases the resulting precipitate.

The experiment demonstrates (a) the inhibiting influence of an excess of antigen so that no precipitate is obtained when the strongest dilution of immune serum (1:5) is mixed with the strongest dilution of antigen (1:10). With higher dilutions of antibody the inhibiting action of antigen is even more evident; with immune serum diluted 1:25 there is no precipitate with antigen diluted 1:100 and with immune serum diluted 1:100 there is no precipitate with antigen diluted 1:1000. (b) The table shows that decreasing quantities of antiserum produce the maximum amount of precipitate when mixed with decreasing amounts of antigen; with a dilution of immune serum of 1:5, for example, maximum precipitation is obtained with antigen diluted 1:1000, with immune serum diluted from 1:20 to 1:50 maximum precipitation is found with antigen diluted from 1:1000 to 1:10,000 and with immune serum diluted 1:100 maximum precipitation is obtained with antigen diluted 1:10,000.

TABLE 1

| ANTI-HORSE SERUM FROM RABBIT | HORSE SERUM | | | | |
|---|---|---|---|---|---|
| | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 |
| 1:5 | 0 | +++ | ++++ | ++ | 0 |
| 1:10 | 0 | + | +++ | ++ | 0 |
| 1:15 | 0 | + | ++ | ++ | 0 |
| 1:20 | 0 | + | ++ | ++ | 0 |
| 1:25 | 0 | 0 | ++ | ++ | 0 |
| 1:50 | 0 | 0 | + | + | 0 |
| 1:100 | 0 | 0 | 0 | + | 0 |
| 1:125 | – | – | – | + | – |
| 1:150 | – | – | – | 0 | – |
| 1:200 | – | – | – | 0 | – |
| 1:300 | – | – | – | 0 | – |

The experiment just described has been repeated with donkey serum and anti-donkey serum from rabbit (no. 289) diluted with normal salt solution.

The table again shows on the one hand that excess of antigen inhibits precipitation and on the other hand that maximum precipitation with diminishing quantities of antibody is produced with diminishing quantities of antigen (the maximum in the table passes to the right from above downward).

TABLE 2

| ANTI-DONKEY SERUM FROM RABBIT | DONKEY SERUM | | | | |
|---|---|---|---|---|---|
| | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 |
| 1:2 | +++ | ++++ | ++++ | +++ | +++ |
| 1:5 | ++ | +++ | ++++ | ++ | + |
| 1:10 | + | ++ | +++ | ++ | + |
| 1:15 | + | ++ | +++ | ++ | + |
| 1:25 | 0 | + | ++ | ++ | + |
| 1:50 | 0 | 0 | + | + | 0 |
| 1:100 | 0 | 0 | 0 | + | 0 |

TABLE 3

| SERUM OF RABBIT IMMUNIZED AGAINST CRYSTALLINE EGG ALBUMIN | SOLUTION OF CRYSTALLINE EGG ALBUMIN (5 PER CENT) | | | | | |
|---|---|---|---|---|---|---|
| | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 | 1:1,000,000 |
| 1:10 | 0 | + | +++ | ++ | + | + |
| 1:25 | 0 | 0 | + | ++ | + | + |
| 1:50 | 0 | 0 | + | ++ | + | 0 |
| 1:100 | 0 | 0 | 0 | + | + | 0 |
| 1:200 | 0 | 0 | 0 | 0 | + | 0 |
| 1:400 | 0 | 0 | 0 | 0 | + | 0 |

Identical results have been obtained when the foregoing experiment has been performed with a purified protein, namely, crystalline egg albumin (5 per cent solution) and serum of a rabbit (no. 418) repeatedly injected with the same substance. Crystalline egg albumin has been prepared by the method of Hopkins and Pinkus (10) and has been recrystallized six times.

The serum employed is unusually strong causing a recognizable precipitate with a 5 per cent solution of crystalline egg albumin diluted with one million times its volume of salt solution,

namely, with 0.00000005 gram dissolved in 1 cc. of salt solution. The relation between strength of antibody and maximum precipitate with antigen is well illustrated; with immune serum diluted 1:10 a maximum precipitate is obtained with antigen diluted 1:1000, with serum diluted 1:200 the maximum precipitation has shifted to a dilution of 1:100,000. This observation suggests the possibility that the maximum precipitate obtained with various strengths may furnish a much more accurate measure of the strength of an immune serum than the point of disappearance of recognizable precipitation with increasing dilution of antigen.

Before these experiments performed *in vitro* with antigen and precipitating sera diluted with normal salt solution are used to

TABLE 4

| ANTI-HORSE SERUM FROM RABBIT DILUTED WITH NORMAL SERUM OF RABBIT | HORSE SERUM DILUTED WITH SERUM OF NORMAL RABBIT | | | |
|---|---|---|---|---|
| | 1:10 | 1:100 | 1:1,000 | 1:10,000 |
| 1:5 | 0 | ++ | +++ | ++ |
| 1:10 | 0 | 0 | ++ | + |
| 1:25 | – | 0 | 0 | + |
| 1:50 | – | 0 | 0 | + |

explain relations found within the body, it is desirable to determine if similar relations are demonstrable when normal serum of rabbit has been substituted for salt solution as a diluent. In the following experiment the attempt is made to determine how antibody and antigen react under conditions similar to those present in the blood.

Excess of antigen inhibits precipitation in the presence of blood serum; and in consequence of this relation precipitates are obtained when high dilutions of antibody are employed only when mixed with high dilutions of antigen. Since the table differs in no essential character from those previously recorded it may be assumed that the relations of antigen and antibody are the same in serum and in salt solution.

### SOLUTION OF PRECIPITATE BY EXCESS OF ANTIGEN

An excess of antigen is capable of dissolving the precipitate formed by the action of the specific precipitating serum used for its production. This solvent action has been noted by several observers (Gay and Rusk). It is possible that it explains the absence of precipitation in the presence of strong solutions of antigen whereas precipitation occurs in the presence of higher dilutions. With this possibility in view the attempt has been made to measure the solution of precipitate by its corresponding antigen. Experiments described in the next section of this paper will show that an accurate measurement of the solubility of a precipitate is possible only when a simple antigen such as crystalline egg albumin is employed.

To test the solubility of precipitate in excess of antigen a constant quantity of immune serum (0.2 cc.) has been added to increasing quantities of antigen (crystalline egg albumin in 5 per cent solution) diluted to a volume of 1 cc. with normal salt solution (tubes A, B, C, D, E and F). The series with the exception of the first tube has been duplicated (tubes b, c, d, e and f). The mixtures are placed in the thermostat and kept there one hour. At the end of this time both sets of tubes are removed. To each tube in the first set is added 1 cc. of salt solution. To each tube in the second set is added an amount of antigen required to increase its total antigen to that of the tube immediately above it in the series, the added volume being brought to one cubic centimeter by addition of normal salt solution. The tubes are again kept in the thermostat during one hour, transferred to the refrigerator and read on the following day.

When the tubes were removed from the thermostat after the first incubation and before addition of antigen to the second series the precipitate present was identical in corresponding tubes (namely B and b, C and c, etc.) and was approximately the same as that found after refrigeration in the first series to which no more antigen was added. Increase of antigen in tube b dissolved the precipitate which had formed so that the end result was identical with that of tube A. Increase of antigen in tube C, representing the maximum precipitation, partially dissolved the precipitate

already formed but did not fully reduce it to the quantity present in tube B. Addition of antigen to tubes d, e and f increased the precipitate as might have been anticipated to the quantities found in tubes C, D and E.

The following experiment is similar to that just recorded; the amount of precipitation present before antigen has been added to tubes b, c, d, e, and f is recorded in the table; comparison between this reading and the final reading obtained after addition of antigen furnishes an index of the solution caused by excess of antigen.

TABLE 5

| TUBE | ANTIGEN ADDED BEFORE INCUBATION | ANTIGEN ADDED AFTER INCUBATION | TOTAL ANTIGEN | RELATIVE AMOUNT OF PRECIPITATE |
|---|---|---|---|---|
| A | 0.1 | | 0.1 | 0 |
| b | 0.01 | 0.09 | 0.1 | 0 |
| B | 0.01 | | 0.01 | + |
| c | 0.001 | 0.009 | 0.01 | ++ |
| C | 0.001 | | 0.001 | +++ |
| d | 0.0001 | 0.0009 | 0.001 | +++ |
| D | 0.0001 | | 0.0001 | + |
| e | 0.00001 | 0.00009 | 0.0001 | + |
| E | 0.00001 | | 0.00001 | Trace |
| f | 0.000001 | 0.000009 | 0.00001 | Trace |
| F | 0.000001 | | 0.000001 | 0 |

The amount of precipitate is dependent upon the total amount of antigen in the mixture and the result is the same if the excess of antigen is added after the precipitate is already formed.

It is noteworthy that the mixture of immune serum and antigen in excess is at times followed immediately by precipitation which is observable if the tubes are examined within a few minutes after the mixture is made. After a half hour this precipitate is dissolved and the so-called inhibition zone is evident.

The next experiment does not differ from those previously described save for the preparation of a third series of mixtures (tubes b,′ c,′ e,′ and f′) to which after precipitate had formed there

was added an excess of antigen which brought the total to 0.1 cc., an amount of antigen which completely inhibits precipitation. In every instance complete solution of the precipitate occurred.

The experiments show that inhibition of precipitation and solution of a precipitate by excess of antigen have the same result so that the amount of precipitate which remains in the mixture depends upon the total amount of antigen which has been added.

TABLE 6

| TUBE | ANTIGEN BEFORE INCUBATION | PRECIPITATION AFTER INCUBATION | ANTIGEN ADDED AFTER INCUBATION | TOTAL ANTIGEN | RELATIVE AMOUNT OF PRECIPITATE |
|---|---|---|---|---|---|
| A | 0.5 | 0 | | 0.5 | 0 |
| b | 0.1 | 0 | 0.4 | 0.5 | 0 |
| B | 0.1 | 0 | | 0.1 | 0 |
| c | 0.05 | 0 | 0.05 | 0.1 | 0 |
| C | 0.05 | 0 | | 0.05 | 0 |
| d | 0.01 | + | 0.04 | 0.05 | 0 |
| D | 0.01 | + | | 0.01 | Trace |
| e | 0.001 | ++ | 0.009 | 0.01 | Trace |
| E | 0.001 | ++ | | 0.001 | ++ |
| f | 0.0001 | ++ | 0.0009 | 0.001 | ++ |
| F | 0.0001 | ++ | | 0.0001 | ++ |
| G | 0.00001 | + | | 0.00001 | + |
| H | 0.000001 | 0 | | 0.000001 | Trace |

An exception may occur when the precipitate approximates the maximum obtainable with a given quantity of antibody; subsequent observations will show that precipitation may leave in the supernatant fluid an excess of antibody which unites with the new increment of antigen and so diminishes its solvent action. The experiments show that inhibition of precipitation by excess of antigen is the result of the ability of the antigen to dissolve the specific precipitate.

Chemical reactions in which a precipitate formed by two solutions is dissolved by an excess of one of them are well known; an example is that in which the precipitate formed by silver nitrate and ammonium hydroxide is dissolved by an excess of the latter. By mixing decreasing quantities of ammonium hydroxide with a given quantity of a solution of silver nitrate an inhibition zone similar to that obtained with antigen and precipitin is obtained.

TABLE 7

| TUBE | ANTIGEN BEFORE INCUBATION | ANTIGEN ADDED AFTER INCUBATION | TOTAL ANTIGEN | RELATIVE AMOUNT OF PRECIPITATE |
|---|---|---|---|---|
| A | 0.1 | | 0.1 | 0 |
| b | 0.02 | 0.08 | 0.1 | 0 |
| B | 0.02 | | 0.02 | 0 |
| c | 0.01 | 0.01 | 0.02 | 0 |
| C | 0.01 | | 0.01 | Trace |
| d | 0.005 | 0.005 | 0.01 | Trace |
| D | 0.005 | | 0.005 | + |
| e | 0.002 | 0.003 | 0.005 | + |
| E | 0.002 | | 0.002 | + |
| f | 0.001 | 0.001 | 0.002 | ++ |
| F | 0.001 | | 0.001 | +++ |
| b′ | 0.02 | 0.08 | 0.1 | 0 |
| c′ | 0.01 | 0.09 | 0.1 | 0 |
| d′ | 0.005 | 0.095 | 0.1 | 0 |
| e′ | 0.002 | 0.098 | 0.1 | 0 |
| f′ | 0.001 | 0.099 | 0.1 | 0 |

## EQUILIBRIUM OF ANTIGEN AND ANTIBODY IN THE PRECIPITIN REACTION

In the foregoing experiments a maximum precipitate is obtained only when the quantity of immune serum is many times that of the antigen used to produce it and this relation exists even though the immune serum is capable of forming a precipitate with high dilutions of antigen, namely, from 1:100,000 to 1:1,000,000 (table 3). In the following experiments the attempt has been

made to determine the relation between precipitate formation and the fate of antigen and antibody in the mixture. For this purpose the supernatant fluid over a precipitate formed by mixtures of antigen and immune serum was tested to determine if these substances persisted in the solution after the reaction had occurred. In the following experiment quantities of the serum of a rabbit immunized against horse serum varying from 0.1 to 2 cc. are brought to a uniform volume of 2 cc. and mixed with 0.005 cc. of horse serum contained in 1 cc. of normal salt solution. After the usual incubation and refrigeration, the supernatant fluid is removed by centrifugalization. It is tested for antibody by mixing with fresh antigen and for antigen by

TABLE 8

| ANTI-HORSE SERUM FROM RABBIT | HORSE SERUM IN 1 CC. OF SALT SOLUTION | PRECIPITATE | SUPERNATANT FLUID TESTED FOR | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Antibody. 0.2 cc. supernatant fluid added to horse serum diluted as follows: | | | | Antigen. 0.2 cc. anti-horse serum from rabbit added to supernatant fluid diluted as follows: | | |
| | | | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:2 | 1:10 | 1:100 |
| *cc.* | *cc.* | | | | | | | | |
| 0.1 | 0.005 | + | 0 | 0 | 0 | 0 | ++++ | +++ | + |
| 0.5 | 0.005 | ++ | 0 | 0 | 0 | 0 | +++ | ++ | 0 |
| 1.0 | 0.005 | ++++ | + | + | ++ | + | ++ | Tr. | 0 |
| 1.25 | 0.005 | ++++ | + | + | ++ | + | + | Tr. | 0 |
| 1.5 | 0.005 | ++++ | + | ++ | ++++ | + | Tr. | 0 | 0 |
| 2.0 | 0.005 | ++++ | ++ | +++ | ++++ | ++ | 0 | 0 | 0 |

addition of immune serum. It is desirable to use various dilutions both of antigen and of supernatant fluid for otherwise antigen may be present in such excess or in such minute quantity that no precipitate is formed (see especially table 1).

In accordance with the observations made by others the table exhibits phases of the reaction (with 1.0, 1.25 and 1.5 cc. of immune serum) in which both antigen and antibody are demonstrable in the supernatant fluid. The maximum precipitate is obtained by mixing 0.005 cc. of horse serum with 1 cc. of immune serum, that is, with 200 times its volume and corresponds with the level at which excess of antibody appears in the supernatant fluid. At and below this level both antigen and antibody are present in the fluid but they fail to unite to form a precipitate.

In the next experiment crystalline egg albumin in 5 per cent solution (approximately that of the proteins of the blood) has been substituted for horse serum.

The maximum precipitation occurs when 0.005 cc. of the solution of crystalline egg albumin is mixed with 2 cc. of immune serum, that is, with 400 times its volume. At this level antibody becomes evident in the supernatant fluid but antibody does not appear until antigen has disappeared so that there is no phase of the reaction in which antigen and antibody coexist in the supernatant fluid.

TABLE 9

| ANTI-EGG ALBUMIN SERUM FROM RABBIT (418) | 5 PER CENT CRYSTALLINE EGG ALBUMIN DILUTED TO 5 CC. | PRECIPITATE | SUPERNATANT FLUID TESTED FOR | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Antibody. 0.5 cc. supernatant fluid with egg-albumin solution diluted as follows: | | | | Antigen. 0.2 cc. anti-egg albumin serum from rabbit added to supernatant fluid diluted as follows: | | |
| | | | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:2 | 1:10 | 1:100 |
| *cc.* | *cc.* | | | | | | | | |
| 0.5 | 0.005 | + | 0 | 0 | 0 | 0 | +++ | ++ | Tr. |
| 1.0 | 0.005 | ++ | 0 | 0 | 0 | 0 | + | 0 | 0 |
| 1.5 | 0.005 | +++ | 0 | 0 | 0 | 0 | + | 0 | 0 |
| 2.0 | 0.005 | ++++ | 0 | 0 | Tr. | Tr. | 0 | 0 | 0 |
| 2.5 | 0.005 | ++++ | 0 | 0 | Tr. | Tr. | 0 | 0 | 0 |
| 3.0 | 0.005 | ++++ | 0 | 0 | + | + | 0 | 0 | 0 |

The next series of three experiments (table 10) were carried out on a somewhat different plan. A constant quantity of immune serum was mixed with increasing quantities of crystaline egg albumin. In every instance the maximum precipitate occurs at the level at which antibody makes its appearance in the supernatant fluid and antibody appears because it is no longer fixed by antigen. One thousand volumes of the serum of Rabbit 420 obtained March 31 were required to fix the antigen in one volume of the solution used in its production whereas two hundred volumes of the serum of rabbit 419 fixed one volume of antigen. The maximum precipitate obtained by mixing immune serum and antigen serves as a measure of the strength of the serum in antibodies.

The experiments with crystalline egg albumin show that antigen and antibody may be simultaneously demonstrable in the clear fluid above a precipitate formed by their action if tests are made with increasing preponderance of antibody at sufficiently close intervals in the zone at and just beyond the level of maximum precipitation. In the tests made with the serum of rabbit 420 of March 26 amounts of serum in

TABLE 10

| Number of rabbits supplying serum, with dates | Anti-egg albumin serum from rabbit | 5 per cent crystalline egg-albumin diluted to 5 cc. | Precipitate | Supernatant fluid tested for: Antibody. 0.5 cc. supernatant fluid with egg albumin solution diluted as follows: | | | | Antigen. 0.2 cc. anti-egg albumin serum from rabbit added to supernatant fluid diluted as follows: | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:2 | 1:10 | 1:100 |
| | cc. | cc. | | | | | | | | |
| 420, March 26 | 1 | 0.05 | 0 | — | 0 | 0 | 0 | +++ | ++ | Tr. |
| | 1 | 0.005 | 0 | — | 0 | 0 | 0 | ++ | + | 0 |
| | 1 | 0.0005 | +++ | — | 0 | Tr. | + | 0 | 0 | 0 |
| 420, March 31 | 1 | 0.05 | Tr. | 0 | 0 | 0 | 0 | ++ | ++++ | ++ |
| | 1 | 0.01 | + | 0 | 0 | 0 | 0 | ++++ | ++ | + |
| | 1 | 0.005 | ++ | 0 | 0 | 0 | 0 | +++ | + | 0 |
| | 1 | 0.001 | ++++ | 0 | 0 | Tr. | Tr. | + | 0 | 0 |
| | 1 | 0.0005 | +++ | 0 | Tr. | Tr. | + | 0 | 0 | 0 |
| 419 | 1 | 0.05 | Tr. | 0 | 0 | 0 | 0 | + | +++ | ++ |
| | 1 | 0.01 | + | 0 | 0 | 0 | 0 | ++ | +++ | + |
| | 1 | 0.005 | ++++ | 0 | 0 | Tr. | 0 | + | + | 0 |
| | 1 | 0.001 | +++ | — | — | ++ | ++ | + | Tr. | 0 |
| | 1 | 0.0005 | ++ | 0 | 0 | +++ | ++ | 0 | 0 | 0 |

consecutive tubes are so widely separated that antigen disappears before antibody makes its appearance but in the two subsequent experiments (with serum of rabbit 420 of March 31 and with serum of rabbit 419) there is a narrow zone in which both antigen and antibody are demonstrable in the fluid above the precipitate. Nevertheless numerous experiments have shown that the two substances are simultaneously demonstrated with far greater readiness when horse serum is employed as antigen. The experiments confirm the observations of Bayne-Jones and

show that crystalline egg albumin like horse serum may be found free in the fluid together with its antibody. The experiments of Bayne-Jones suggesting that crystalline egg albumin, prepared by the usual methods, is not a simple substance for anaphylactic tests, indicate that the presence of globulin is not wholly excluded. The assumption of a multiplicity of antigens, supported by von Dungern and by Weil, explains the apparent overlapping of tests for antigen and antibody. The readiness with which antibodies are produced when antigen is injected in small amounts into rabbits and the extreme delicacy of the precipitin test would facilitate the demonstration of antibodies caused by failure to completely purify the crystaline egg albumin.

One consideration which has an important bearing on the foregoing discussion has heretofore been overlooked. In the so called zone of inhibition, where precipitate is held in solution by excess of antigen, antigen and antibody are side by side in the same fluid but do not combine to form a precipitate. This relation is very obvious where antigen is in such excess that no precipitate is formed but exists in less degree whenever with increasing preponderance of antibody the latter is insufficient to form the maximum precipitate obtainable with a given quantity of antigen. In these mixtures the presence of antigen is readily demonstrated by addition of immune serum but, since the specific precipitate is already soluble in the excess of antigen in the mixture, addition of antigen will not result in the formation of a precipitate. Two conditions are distinguishable (a) antigen and its own antibody coexist in a solution with no formation of precipitate when antigen is in such excess that it holds the precipitate in solution. Addition of antibody will demonstrate the presence of antigen but addition of antigen will not form a precipitate because it is already present in excess. (b) When a precipitate is formed by antigen and antibody, in certain phases of the reaction both antigen and antibody may be demonstrable in the supernatant fluid by addition of antibody on the one hand or antigen on the other. This condition is present when the precipitate formed approximates the maximum obtainable with a given quantity of antigen.

## CONCLUSIONS

Excess of antigen dissolves the precipitate formed by action of precipitin and its antigen.

Inhibition of specific precipitation (inhibition zone) in the presence of high concentration of antigen is caused by the solvent action of excess of antigen.

Proteins of the blood serum do not prevent precipitation and the reaction has the same characters whether dilutions of antigen and antibody are made in salt solution or in normal blood serum.

When a consistent quantity of antigen is mixed with increasing quantities of immune serum the maximum amount of precipitate is found when antigen is mixed with several hundred times its volume of immune serum, the number of volumes depending upon the strength of the serum. An excess of antigen added to the mixture becomes demonstrable in the supernatant fluid. This relation furnishes a measure of the strength of the immune serum determined in multiples of the volume of antigen used for the test.

The presence of antigen and antibody in the supernatant fluid over a precipitate obtained by adding to antigen (horse serum, egg white, etc.) an amount of antibody in excess of that required to produce the maximum amount of precipitate is best explained by the assumption of a multiplicity of antigens in such complex mixtures as blood serum or egg white. Crystalline egg albumin is an almost pure antigen but contains in very small amount extraneous antigen capable of forming precipitin.

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# OBSERVATIONS ON THE PROTECTIVE ACTION OF NORMAL SERUM IN EXPERIMENTAL INFECTION WITH BACILLUS DIPHTHERIAE

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In carrying out test inoculations in guinea-pigs with *B. diphtheriae* and diphtheroid bacilli for purposes of identification, where control animals were injected with the particular strain *plus* a certain amount of diphtheria antitoxin, it was noted that these animals were protected against lethal doses of *B. diphtheriae* by normal horse serum as well as by the specific immune serum. Though this observation did not involve any quantitative comparisons between the effect of the normal and the immune serum, the fact that normal horse serum should act protectively in the same way as the serum of a specifically immunized animal seemed of considerable importance and suggested the further investigation of the phenomenon. This normal serum effect is also of general interest in relation to "non-specific therapy" where alien protein (e.g., animal serum, bacterial protein, etc.) injected parenterally is found to exert a favourable influence on the course of various infections in a non-specific manner.

With a view, therefore, to analyzing the effect, a series of experiments were carried out which fully established the original observation and yielded further information of some theoretic and practical interest.

The protective and curative action of normal horse serum in *B. diphtheriae* infection and intoxication has also been studied by other workers.

Kolle and Schlossberger concluded that in guinea-pigs infected with *B. diphtheriae* normal horse serum had a limited

curative effect, which was in no way comparable with that of an antitoxic serum, and in the case of animals injected with diphtheria toxin, had a certain "delaying" action but was not definitely curative. They stated that the effect was "non-specific, resistance increasing, stimulating" but did not offer any further explanation of their results. Kraus and Sordelli claimed that normal horse serum contains antitoxin to diphtheria toxin and in virtue of this exerts a "preventive" effect against *B. diphtheriae* and its toxin. Cowie and Greenthal showed that 1 cc. of normal horse serum injected subcutaneously and intravenously in guinea-pigs simultaneously with diphtheria toxin always protects against 1 M.L.D. and may neutralize even 8 M.L.D.; they showed that the activity of the serum depends on the proteins and not on the alcohol-soluble fraction. They regarded the protective power as not entirely due to a natural diphtheria antitoxin.

In the writer's original experiments guinea-pigs of approximately 350 grams weight were injected subcutaneously with 2 cc. of a two days' bouillon culture of virulent strains of *B. diphtheriae* and at the same time 5 to 10 cc. of normal horse serum were injected subcutaneously at a different site from that of inoculation; these animals survived while animals of similar weight injected with the same amount of the same cultures died within twenty-four to forty-eight hours, showing at autopsy the characteristic effects of *B. diphtheriae* inoculations. Various strains and different specimens of normal horse serum gave the same results.

For the detailed investigation, particular strains of *B. diphtheriae* isolated from typical cases of throat diphtheria were used.

### DEGREE OF PROTECTIVE ACTION OF NORMAL HORSE SERUM

In order to ascertain quantitatively the degree of protection conferred by normal serum, guinea-pigs of approximately equal weight were injected subcutaneously with a fixed quantity of a *B. diphtheriae* bouillon culture and at the same time with varying amounts of horse serum; the minimum lethal dose of the culture was also estimated in parallel experiment (table 1).

Thus 2 cc. of serum protected against at least 12 lethal doses of the culture (i.e., 1 cc. serum against 6 M.L.D.).

TABLE 1

*Guinea-pigs approximately 400 grams weight injected subcutaneously in flank with 3 cc. of a two days' bouillon culture B. diphtheriae "strain 3"; varying amounts of normal horse serum injected subcutaneously in back*

| NUMBER | CULTURE | AMOUNT OF SERUM | RESULT |
|---|---|---|---|
| | | cc. | |
| 1 | | 6.0 | Survived |
| 2 | | 5.0 | Survived |
| 3 | | 4.0 | Survived |
| 4 | | 3.0 | Survived |
| 5 | *B. diphtheriae*.......................... | 2.0 | Survived |
| 6 | | 1.0 | Died 6th day |
| 7 | | 0.5 | Died 4th day |
| 8 | | 0.25 | Died 3d day |
| 9 | | 0.1 | Died 4th day |
| 10 | | No serum | Died 2d day |

*Minimum lethal dose estimation. Guinea-pigs approximately 400 grams weight; same culture injected subcutaneously*

| NUMBER | CULTURE | AMOUNT OF SERUM | RESULT |
|---|---|---|---|
| 1 | | 1.5 | Died 2d day |
| 2 | | 1.0 | Died 2d day |
| 3 | *B. diphtheriae*.......................... | 0.5 | Died 4th day |
| 4 | | 0.25 | Died 7th day |
| 5 | | 0.1 | Survived |
| 6 | | 0.05 | Survived |

## PROPHYLACTIC AS OPPOSED TO CURATIVE EFFECT

It was found that normal serum though protective or prophylactic was not curative; thus, to elicit the antagonistic effect the serum had to be injected without delay after the introduction of the infecting organisms; if an interval of two hours or longer was allowed to elapse before the serum was injected even when 5 times the protective dose was used the animals died (table 2).

## THERMOSTABILITY OF THE ACTIVE SUBSTANCE IN THE SERUM

In thermostability, the active principle of the serum corresponded generally to antitoxin and other antibodies. Horse

TABLE 2

*Guinea-pigs approximately 400 grams weight injected subcutaneously in flank with 3 cc. two days' bouillon culture B. diphtheriae "strain 3"; 10 cc. normal horse serum injected subcutaneously in back at varying intervals after injection of the culture*

| NUMBER | CULTURE + NORMAL HORSE SERUM | TIME | RESULT |
|---|---|---|---|
| 1 | | Simultaneously | Survived |
| 2 | | After 2 hours | Died 3d day |
| 3 | *B. diphtheriae* + serum.............. | After 4 hours | Died 2d day |
| 4 | | After 24 hours | Died 2d day |
| 5 | *B. diphtheriae,* no serum.............. | | Died 2d day |

TABLE 3

*Guinea-pigs approximately 450 grams weight injected subcutaneously in flank with 3 cc. of a two days' bouillon culture of B. diphtheriae "strain 2"; normal horse serum injected subcutaneously in back at same time*

| NUMBER | CULTURE + NORMAL HORSE SERUM | RESULT |
|---|---|---|
| 1 | *B. diphtheriae* + 10 cc. unheated serum. | Survived |
| 2 | *B. diphtheriae,* no serum.............. | Died 2d day |
| 3 | *B. diphtheriae* + 10 cc. serum 57°C. 1 hour.............................. | Survived |
| *4 | *B. diphtheriae* + 50 cc. serum diluted 1 in 5 with normal saline and heated at 70°C. one-half hour.............. | Died 2d day |
| *5 | *B. diphtheriae* + 60 cc. serum diluted 1 in 5 and heated at 90°C. one-half hour.............................. | Died 2d day |
| *6 | *B. diphtheriae* + 50 cc. serum diluted 1 in 5 and heated at 100°C. for 5 minutes............................ | Died in twenty-four hours showing more marked lesions than other animals—marked hemorrhagic inflammatory edema at site of inoculation with marked hemorrhages in suprarenals, lungs and intestinal wall |

* The serum was diluted to prevent coagulation on heating. 50 cc., 60 cc. = diluted serum.

serum heated for one hour at 57°C. was still protective but was inactivated by exposure at 70°C. for one-half hour and at higher temperatures (table 3). The thermostability at 57°C. excluded the possibility of the active substance being of complement nature.

### EXPERIMENTS WITH RABBITS

Using rabbits as the test animal, horse serum was found to exert the same protective action against *B. diphtheriae* as in the case of guinea-pigs; the particular effect was not therefore limited to *B. diphtheriae* infection in one species of animal only. Table 4 exemplifies one of these experiments.

TABLE 4

*Rabbits of equal weight injected subcutaneously with 4 cc. of a three days' bouillon culture of B. diphtheriae "strain 3"*

| NUMBER | B. DIPHTHERIAE CULTURE | RESULT |
|---|---|---|
| 1 | + 20 cc. normal horse serum injected subcutaneously at different site | Survived |
| 2 | No serum | Died 3d day |

### PROTECTIVE ACTION OF THE SERUM OF DIFFERENT ANIMALS

The serum from various animal species (ox, sheep, man, rabbit, cat) was found to confer the same protection as horse serum; in the experiments of other workers on this subject the phenomenon has been studied from the point of view apparently of its being related to horse serum only; the effect is however not characteristic of the serum of only one species of animal (table 5).

The question then arose as to whether the serum of one guinea-pig injected parenterally into another guinea-pig would protect the latter against *B. diphtheriae* infection. To ascertain this a number of experiments were carried out but with variable results; in some cases complete protection was obtained, in others while the infected animals succumbed to the dose given there was a distinct delaying of the lethal effect (table 6). Thus it was apparent that though protection by normal serum from an alien species was the general rule, the serum of the same species was not without a similar effect.

TABLE 5

*Guinea-pigs approximately 450 grams weight injected subcutaneously in flank with 3 cc. of a two days' bouillon culture of B. diphtheriae "strain 2"; serum of various animals injected subcutaneously in back at same time*

| NUMBER | CULTURE + SERUM | RESULT |
|---|---|---|
| 1 | + 10 cc. ox serum | Survived |
| 2 | + 10 cc. sheep's serum | Survived |
| 3 | + 8 cc. human serum | Survived |
| 4 | *B. diphtheriae* + 10 cc. cat's serum | Survived |
| 5 | + 6 cc. rabbit's serum | Survived |
| 6 | + 3 cc. rabbit's serum (55°C.) | Survived |
| 7 | + 10 cc. horse serum | Survived |
| 8 | no serum | Died 2d day |

TABLE 6

*Guinea-pigs approximately 400 grams weight injected subcutaneously with 3 cc. two days' bouillon culture of B. diphtheriae; serum from other guinea pigs injected subcutaneously at a different site*

| NUMBER | CULTURE + GUINEA-PIG'S SERUM | RESULT |
|---|---|---|
| 1 | (a) *B. diphtheriae* ("strain 2") + 6 cc. serum | Died 6th day |
| | (b) Control (no serum injected) | Died 2d day |
| 2 | (a) *B. diphtheriae* ("strain 2") + 8 cc. serum (55°C.) | Survived |
| | (b) Control | Died 2d day |
| 3 | (a) *B. diphtheriae* ("strain 2") + 10 cc. serum (from 2 animals; sera pooled) | Died 19th day |
| | (b) Control | Died 2d day |
| 4 | (a) *B. diphtheriae* ("strain 3") + 5 cc. serum (from 2 animals) and 5 cc. following day | Survived |
| | (b) Control | Died 2d day |
| 5 | (a) *B. diphtheriae* ("strain 3") + 10 cc. serum | Survived |
| | (b) Control | Died 2d day |
| 6 | (a) *B. diphtheriae* ("strain 3") + 10 cc. serum | Died 5th day |
| | (b) Control | Died 2d day |
| 7 | (a) *B. diphtheriae* ("strain 3") + 20 cc. serum | Died 8th day |
| | (b) Control | Died 2d day |
| 8 | (a) *B. diphtheriae* ("strain 3") + 10 cc. serum | Survived |
| | (b) Control | Died 3d day |

It is a phenomenon of considerable interest that the serum of one individual of an animal species, which is highly susceptible to experimental *B. diphtheriae* infection, should be capable of affording some protection, when injected parenterally in certain amounts, to another individual of the same species infected with the particular organism.

## EXPERIMENTS WITH OTHER PROTEINS; BACTERICIDAL EXPERIMENTS

Experiments were carried out with egg-albumen, milk and peptone, to determine whether the parenteral injection of these proteins would confer any protection as in the case of the serum proteins. The results were uniformly negative.

*In vitro* bactericidal tests were also made to ascertain whether horse serum (in varying amounts) along with fresh guinea-pig's serum as complement, exerted a specific bactericidal action on *B. diphtheriae* but no such effect could be demonstrated, thus excluding the possibility of the protection being due to a normal bactericidal immune body in horse serum.

## EXPERIMENTS WITH DIPHTHERIA TOXIN

Experiments were then carried out to ascertain whether the protective effect was due to a natural antitoxic action on the part of the serum injected, though it seemed difficult to explain on this basis the action of guinea-pig's serum in the case of infection in the same species.

A powerful toxin was prepared from *B. diphtheriae* "strain 3." It was found that 10 cc. of horse serum was protective against 10 M.L.D. of the toxin (1 cc. against 1 M.L.D.), whereas 1.5 cc. of diphtheria antitoxin representing 1000 immunity units protected against 10,000 M.L.D. The minimum lethal dose was estimated in parallel experiments along with the protective tests (table 7).

A similar series of experiments were carried out in which the pooled serum of three guinea-pigs was substituted for horse serum (table 8).

The parenteral injection of 10 cc. of guinea-pig's serum had apparently no protective action in guinea-pigs against *B. diphtheriae* toxin.

TABLE 7

*Guinea-pigs 250 grams weight injected subcutaneously in flank with varying doses of diphtheria toxin; at the same time injected subcutaneously in back with 10 cc. normal horse serum*

| NUMBER | AMOUNT OF TOXIN | AMOUNT OF SERUM | RESULT |
|---|---|---|---|
| | *cc.* | *cc.* | |
| 1 | 0.1 | 10 | Died 2d day |
| 2 | 0.05 | | Died 2d day |
| 3 | 0.01 | | Died 7th day |
| 4 | 0.005 | | Died 12th day |
| 5 | 0.001 | | Survived |
| 6 | 0.0005 | | Survived |
| 7 | 1.0 | 1000 immunity units diphtheria toxin | Survived |
| | *M. L. D. estimation* | | |
| 1 | 0.1 | | Died 2d day |
| 2 | 0.01 | | Died 2d day |
| 3 | 0.001 | | Died 2d day |
| 4 | 0.0005 | | Died 2d day |
| 5 | 0.0001 | | Died 3d day |
| 6 | 0.00005 | | Survived |
| 7 | 0.00001 | | Survived |

M. L. D. = 0.0001 cc.

*Note:* The small doses of toxin 0.05 cc. to 0.00001 cc. were measured by preparing decimal dilutions of the toxin 1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and injecting the appropriate quantities of these dilutions.

TABLE 8

*Guinea-pigs 250 grams weight injected subcutaneously in flank with varying doses of diphtheria toxin; at the same time 10 cc. of guinea-pigs serum (from 3 animals, pooled) injected subcutaneously in back*

| NUMBER | AMOUNT OF TOXIN | AMOUNT OF SERUM | RESULT |
|---|---|---|---|
| | *cc.* | *cc.* | |
| 1 | 0.001 | 10 | Died 2d day |
| 2 | 0.0001 | | Died 2d day |
| 3 | 0.00005 | | Survived |
| 4 | 0.00001 | | Survived |
| | *Lethal dose estimation* | | |
| 1 | 0.001 | | Died 2d day |
| 2 | 0.0001 | | Died 2d day |
| 3 | 0.00005 | | Survived |
| 4 | 0.00001 | | Survived |

### THE OCCURRENCE OF LOCAL LESIONS IN ANIMALS SURVIVING AFTER PROTECTION BY NORMAL SERUM

An interesting feature of these experiments was the occurrence of a marked local lesion in animals surviving after protection by normal serum against *B. diphtheriae* or its toxin.

After two to three days a well marked subcutaneous indurated swelling with surrounding inflammatory oedema developed at the site of injection of the organisms or toxin; the overlying skin then became necrotic and sloughed, leaving a punched-out ulcer with a grey sloughing base; the lesion attained its full development in eight to ten days and then slowly healed; in one instance the lesion involved the deeper tissues and led to perforation of the whole abdominal wall.

It was found that diphtheria antitoxin prevented the occurrence of the local lesion as well as the general effects.

This type of lesion occurred in rabbits protected by normal serum as well as in the case of guinea-pigs.

In the infection experiments no diphtheria bacilli could be detected in the ulcers after sloughing of the skin, either by microscopic examination or culture. On the other hand, in experiments where the injection of guinea-pig serum delayed the lethal effect of *B. diphtheriae* for several days (e.g., eight days, nineteen days, v. table 6) and similar sloughing ulcers developed at the site of inoculation, *B. diphtheriae* were present in the lesion.

Thus, the parenteral injection of normal serum though protecting against the lethal result of infection with *B. diphtheriae* had no influence on its local toxic action. It was apparent also that *B. diphtheriae* did not persist in the tissues of animals fully protected by serum.

In unprotected animals the local lesion of the type described is not commonly met with, as animals infected with a sufficient dose die within a few days; for the full development of the local effect a period of eight to ten days is required, and it was apparent that the occurrence of this particular lesion in protected animals was simply dependent on the survival of the animal. In rabbits,

which are less susceptible to experimental *B. diphtheriae* infection and resist larger doses than guinea-pigs, it has been noted that sublethal doses may produce similar marked local lesions apart from any serum protection.

## DISCUSSION

These observations represent a further contribution to the study of non-specific immunization by parenteral injection of normal serum.

While it is possible that in the case of horse serum a natural antitoxin may contribute to the protection noted, and it is of importance that the active substance in the serum corresponds to antibodies in thermostability, the action of the serum in bringing about some other antagonistic effect must be considered; this effect apparently does not depend on the transference of bactericidal complement or a natural bactericidal antibody; the serum is protective against infection apart from intoxication: thus the organisms are absent from the local lesion; it might be claimed, however, that the antitoxic factor contributes to the elimination of the bateria by rendering them more susceptible to phagocytosis through neutralization of their toxin. It is to be noted also that normal serum is not antitoxic as regards the local toxic effects, whereas diphtheria antitoxin protects animals against local as well as general effects. The fact that the parenteral injection of the serum of one guinea-pig into another exerts a "delaying" effect in diphtheria infections and even complete protection, though this species has little natural immunity to *B. diphtheriae* and no natural antitoxin can be demonstrated in the serum, clearly indicates that some mechanism other than that of a natural antitoxin or other antisubstance transferred with the serum, plays the important part in increasing the resistance of the animal.

These experiments are therefore of interest in their bearing on the general question of "non-specific immunity" and provide a further illustration of how the resistance of the tissues to a particular organism may be enhanced by the parenteral injec-

tion of normal serum. The results, however, clearly exclude the possibility that normal horse serum could be as effective a therapeutic agent as a potent diphtheria antitoxin.

## CONCLUSIONS

1. In guinea-pigs experimentally infected with *B. diphtheriae* normal serum (horse, ox, sheep, cat, rabbit, human), injected subcutaneously at the same time as the inoculation, exerts a definite protective action.

2. Two cubic centimeters of normal horse serum may protect in this way against 12 M.L.D. of a *B. diphtheriae* culture.

3. No protection occurs if the serum injection is delayed for two hours after the inoculation.

4. The activity of the serum persists at 57°C. but is lost at 70°C. and higher temperatures.

5. The serum of one guinea-pig injected subcutaneously into another is either protective to the latter experimentally infected with *B. diphtheriae* or at least exerts a definite delaying effect on the course of the infection.

6. Normal horse serum is also similarly protective in guinea-pigs injected with diphtheria toxin; 10 cc. of serum may protect against 10 M.L.D.

7. The serum of one guinea-pig is not protective in the case of another injected with diphtheria toxin.

8. In the case of animals surviving after protection by normal serum a marked local lesion develops at the site of inoculation.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## I. VASO-MOTOR REACTIONS IN THE ISOLATED CANINE LIVER[1]

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It has been shown (1) and is now generally accepted (2, 3, 4) that the sudden, pronounced fall in arterial blood pressure, the characteristic feature of acute anaphylactic shock in dogs, is due to reactions taking place in the liver.

Several theories have been suggested as to the possible nature of these hepatic reactions. The initial theory (1) pictured the reaction as an explosive formation or liberation of vaso-dilator substances by the hepatic parenchyma. According to this theory the fall in arterial blood pressure is due to the action of these hepatic products on the systemic blood vessels.

This theory is in accord with the pharmacological analysis of Biedl and Kraus (5), who found the immediate cause of the blood pressure fall in canine anaphylaxis to be a paralysis of the vaso-constrictor nerve endings. The initial theory has also received support from the observations of Nolf (6), confirmed by Weil and Eggleston (7). Nolf found that the isolated anaphylactic liver, perfused with a mixture of anaphylactic blood and specific foreign protein, reacts with the explosive formation or liberation of antithrombic substances.

Weil and Eggleston failed, however, in their subsequent attempt to demonstrate the assumed vaso-dilator substance. They withdrew blood from dogs dying from anaphylactic shock and introduced this blood intravenously into normal dogs. The

[1] Presented before the joint meeting of the American Association of Immunologists and the American Association of Pathologists and Bacteriologists, Washington, D. C., May 2, 1922.

transfused dogs showed no depressor symptoms half an hour later, after recovery from the anesthetic. These workers, however, did not test blood taken during the initial stages of anaphylaxis, nor did they employ kymographic methods. By the method they employed a transient non-fatal type of anaphylaxis can not be detected. The clinical symptoms of non-fatal shock are readily masked by an anesthetic. Weil and Eggleston's negative result, therefore, can not be taken as evidence against the initial theory.

In opposition to this chemical theory, there have been proposed several purely mechanical theories. The first of these mechanical theories, suggested by Meltzer (8), pictures the fall in systemic blood pressure as due to decreased systemic blood volume resulting from hepatic vaso-dilation. Weil and Eggleston (7) subsequently measured the hepatic blood volume during shock and found this blood volume markedly increased. This increase, however, they believed was not due to local vaso-dilation but to local passive congestion. They conceived this passive congestion to be caused by a mechanical narrowing of the hepatic capillaries, as a result of swelling and vacuolization of the hepatic parenchyma.

A modification of the theory of Weil and Eggleston has been recently proposed by Simons (9). Simons believes that the passive congestion of the liver in anaphylactic and peptone shock is not due to parenchymatous swelling but to local veno-constriction. He bases this belief on histological grounds, the finding of unusually markedly developed smooth muscle coats in the canine hepatic veins. A somewhat similar conception was previously applied by Cannon (10) to account for the splanchnic engorgement in surgical shock.

Simons' hepatic veno-constriction theory as to the underlying mechanism in canine anaphylaxis is of fundamental importance. If true, it tends to unify the apparently dissimilar anaphylactic phenomena in different animal species, by reducing them all to a single underlying reaction. According to this theory, the characteristic symptoms of acute anaphylaxis in different animal species depend upon differences in distribution of non-striated

muscle in these species. Thus, Simons conceives the characteristic features in guinea-pig anaphylaxis to be due to the exceptionally highly developed musculature of the guinea pig bronchi; the characteristic picture in rabbits to depend upon the exceptionally highly developed smooth muscle coats of the pulmonary arteries of rabbits; the characteristic manifestations in dogs to result from the exceptionally highly developed musculature of the canine hepatic veins; and the characteristic features in man possibly to depend upon the exceptionally highly developed musculature of the human adrenal veins (11). We have therefore selected Simons' veno-constrictor theory as the initial theory to be tested.

## TESTS OF SIMONS' HEPATIC VENO-CONSTRICTOR THEORY

This theory was tested by perfusion experiments with isolated organs. Canine livers were perfused with Ringer's solution, under conditions of constant pressure and temperature, and the rate of flow of the perfusion fluid noted. The Ringer's solution was then changed to Ringer's solution containing vaso-constrictor substances, and the resulting changes in rate of flow determined.

This method of studying vaso-constrictor phenomena gives very sharp reactions with certain tissues. Thus, the rate of perfusion through the isolated canine kidney (*A*, fig. 1) is reduced 90 per cent within two minutes as a result of the combined action of adrenalin chloride and barium chloride. The rate of flow through an isolated canine intestinal loop (*B*, fig. 1) is reduced 50 per cent within three minutes by adrenalin chloride alone.

*a. Isolated canine lungs.* In making these hepatic tests, we have used the isolated canine lungs as the control organ. To make these pulmonary perfusions the ascending aorta and inferior vena cava were ligated, an afferent canula was placed in the superior vena cava, and an efferent canula in the left auricular appendage. The lungs were then inflated to their mid-respiratory volume. The perfusion pressure varied from 20 mm. Hg. to 40 mm. Hg. in different experiments, 25 mm. Hg. being

used in most of the tests. Typical data from such pulmonary perfusions are shown in figure 2.

FIG. 1. VASO-CONSTRICTOR REACTIONS IN THE ISOLATED KIDNEY AND ISOLATED INTESTINAL LOOP

Cross-hatched areas represent perfusion flow per minute with Ringer's solution. Black areas show flow per minute (or half minute) with Ringer's solution containing vaso-constrictor substances. Star (*) shows time of changing perfusion clamps. Temperature, 38°C.; perfusion pressure, 80 mm. Hg.

*A* = Perfusion of canine kidney with a mixture of 0.0002 per cent adrenalin chloride and 0.02 per cent Barium chloride.

*B* = Perfusion of canine intestinal loop with 0.0002 per cent adrenalin chloride.

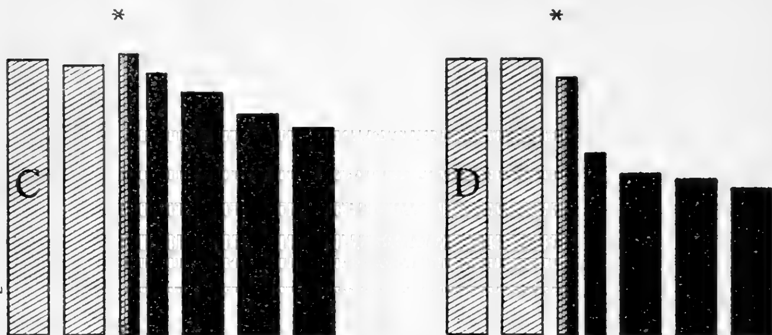


FIG. 2. VASO-CONSTRICTOR REACTIONS IN THE ISOLATED LUNGS

*C* = Perfusion with a mixture of 0.0002 per cent adrenalin chloride and 0.02 per cent barium chloride.

*D* = Perfusion with 0.01 per cent Vaughan's protein split-product in non-alkaline Ringer's solution.

As noted by previous workers (12), but slight decreases in the rate of perfusion flow are demonstrable in the isolated lungs

with the usual vaso-constrictor agents. Thus, the combined action of adrenalin chloride and barium chloride (*C*, fig. 2) reduces the rate of flow but 15 per cent. Marked vaso-constriction, however, can be produced with stronger vaso-constrictor

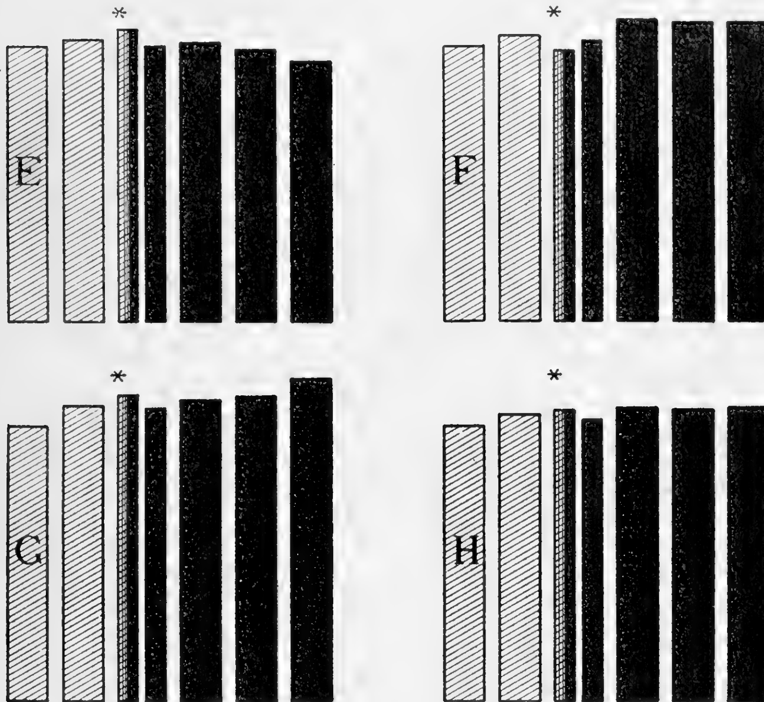


FIG. 3. VASO-CONSTRICTOR TESTS WITH ISOLATED LIVERS

*E* = Perfusion with a mixture of 0.0002 per cent adrenalin chloride and 0.02 per cent barium chloride.

*F* = Perfusion with 0.01 per cent Vaughan's protein split-product in non-alkaline Ringer's solution.

*G* = Perfusion with 0.03 per cent ergotin.

*H* = Composite picture of six hepatic perfusions with the above agents.

substances. Thus, 0.03 per cent ergotin or 0.01 per cent Vaughan's protein split-product (*D*, fig. 2) reduces the rate of pulmonary perfusion fully 35 per cent by the end of one minute, increasing to over 50 per cent by the end of three minutes.

*b. Isolated canine liver.* The agents found to give demonstrable vaso-constriction in the isolated lungs were now tested on isolated canine livers. To make these tests the abdominal vena cava and hepatic artery were ligated, an afferent canula was placed in the portal vein, and an efferent canula in the vena cava immediately above the diaphragm. The perfusion pressure varied from 13 mm. Hg. to 28 mm. Hg. in different experiments, 20 mm. Hg. being used in most of the tests. Typical data from such hepatic perfusions are shown in figure 3.

None of the usual vaso-constrictor agents, such as barium chloride and adrenalin chloride (*E*, fig. 3) produced demonstrable veno-constriction in this organ. Even such powerful vaso-constrictor substances as 0.03 per cent ergotin (*F*, fig. 3) and 0.01 per cent Vaughan's protein split-product (*G*, *H*, fig. 3) gave no suggestion of decreased perfusion flow.

From these observations we conclude that it is not possible for the musculature of the hepatic veins to produce a demonstrable increase in the resistance to blood flow through the liver, even when this musculature is thrown into maximum contraction. Simons' hepatic veno-constrictor theory as to the fundamental reaction in canine anaphylaxis, therefore, does not appear to represent a physiologic possibility.

## SUMMARY

1. Simons' theory as to the nature of the fundamental reaction in canine anaphylaxis was tested by perfusion methods with isolated tissues.

2. Distinct vaso-constriction can be demonstrated in the canine lungs by perfusion with a mixture of barium chloride and adrenalin chloride, and marked vaso-constriction by perfusion with ergotin or with Vaughan's protein split-product.

3. No veno-constriction can be demonstrated in canine livers however by perfusion with any of these agents.

4. Simons' hepatic veno-constrictor theory, therefore, does not appear to represent a physiologic possibility.

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# THE RELATION OF ANTIGEN TO ANTIBODY (PRECIPITIN) IN THE CIRCULATING BLOOD

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The experiments described in this paper have been undertaken with the purpose of determining in what degree the fate of antigen introduced into an immune animal differs from that of the same antigen introduced into a normal animal. The studies of many observers indicate that injected antigen appears in the circulating blood both of normal and immune animals and some have found no difference between the two.

An antigen such as horse serum or egg white which has entered the blood of a normal or of an immunized animal is demonstrable by the addition of immune serum from another animal even though, in the case of the immunized animal, its serum exhibits a high precipitin titer when brought into contact with the antigen against which the animal is immunized. The reactions indicate that free antigen and free antibody are present in the circulating blood but they do not unite to form a precipitate. Upon the basis of these observations some have reached the conclusion that specific precipitation does not occur in the living body. A preceding article (This Journal, 1923, 8, 19) has described the characters of the precipitin reaction *in vitro;* in the present article the attempt is made to show that the changes which occur in the body are explained by those observable *in vitro.*

Soon after the injection of egg white into the subcutaneous tissue this substance is demonstrable by the precipitin reaction within the urine (Ascoli (1)). In the immunized as well as in the normal animal the protein has been found in the blood by Obermeyer and Pick (2) two hours after its injection. No dif-

ference between normal and immune animals was found by Hamburger (3) though neither the method nor the degree of immunization is described. In two normal and in two immunized rabbits a precipitin reaction for egg white appeared in the serum two hours after subcutaneous injection, reached a maximum after twenty-four hours, persisted until the third day and had disappeared on the fourth.

In normal rabbits which had received 5 cc. of horse serum intravenously Uhlenhuth and Weidanz (4) found horse serum in the blood fifteen days after injection. Eight days after the injection slight antibody formation was demonstrable and with the gradual increase of precipitin there was corresponding diminution of antigen. With antisera which as the result of repeated injections of horse serum exhibited a tolerably high precipitin titer free antigen was nevertheless present a long time after the last injection.

In immunized rabbits Bayne-Jones (5) found edestin or crystalline egg albumin in the circulating blood during forty-eight hours after its injection although precipitin maintaining a high titer was present at the same time.

The coexistence of antigen and antibody in the blood of rabbits immunized against horse serum ten days after the last injection was observed by Ionesco-Mihaiesti (6). These animals had received from 40 to 70 cc. of horse serum into the peritoneal cavity within a period of twenty days. The rabbit's serum which contained antigen gave a precipitin reaction in dilution of 1:1000 and caused active sensitization of guinea pigs to anaphylactic intoxication with horse serum.

Rabbits highly immunized against horse serum and subsequently reinjected intravenously with 1 cc. of horse serum, according to Gay and Rusk (7), retain a demonstrable quantity of antigen in the blood during at least twenty-four hours and under exceptional circumstances during a longer period even though the serum contains precipitin evident in dilutions varying from 1:1000 to 1:10,000. This serum containing both antigen and antibody does not fix complement and in contradiction to the observation of Ionesco-Mahaiesti has failed to sensi-

tize guinea pigs actively to subsequent intoxication with horse serum.

Hamburger and Moro (8) found antigen and precipitin present in the blood of children who suffered with serum disease following the administration of diphtheria antitoxin. As precipitin increased in concentration antigen decreased. Longcope and Rackemann (9) studied the relation of circulating antibodies to serum disease in patients who had received from 180 to 360 cc. of antipneumococcus horse serum during the course of lobar pneumonia. The average time between injection of serum and onset of serum disease was four to eight days; precipitins were demonstrable three or four days after the onset of serum sickness. In patients who failed to develop serum disease formation of antibodies was delayed or impaired. In eight individuals studied by Longcope and Mackenzie (10) antigen persisted in the serum during a period from eighteen to thirty-nine days after injection, precipitins were present and serum disease occurred. In a second group of patients antigen persisted from forty-nine to sixty-seven days or longer, precipitin formation was of short duration or entirely absent and serum disease was mild or absent. In one additional instance antigen persisted more than seventy five days, precipitin was found and there was severe serum sickness. In one group of patients following the injection of large quantities of horse serum Mackenzie and Leake (11) observed the formation of precipitins, the occurrence of serum disease and disappearance of antigen approximately coincident with subsidence of symptoms. In a second group there was no serum disease, little or no precipitin formation and persistence of antigen during the period of observation. In a third intermediate group the relationships noted in the two groups were not present.

## THE PERSISTENCE OF ANTIGEN IN THE BLOOD AFTER ITS INJECTION INTO NORMAL ANIMALS

When an antigen such as egg white or horse serum is administered to a rabbit by subcutaneous, intramuscular or intraperitoneal injection it appears in the blood a short time later and is recognizable by the precipitin test during a period of days the duration of which differs considerably in different animals.

Rabbit 291 received 1 cc. of horse serum injected into the subcutaneous tissue. The blood serum before and forty-eight hours after injection was tested by mixing 1 cc. diluted 1:2, 1:5, 1:10, 1:100 and 1:1000 with 0.2 cc. of antihorse serum from a rabbit so strongly immunized aginst horse serum that it produced a precipitate with horse serum diluted 1:20,000.

When 0.2 cc. of the antihorse serum from rabbit (no. 273) used in the foregoing experiment is tested with 1.0 cc. of dilutions of antigen

TABLE 1

*Antigen (horse) in blood serum after its injection into normal rabbit*

| DILUTION | PRECIPITATE WITH 0.2 CC. ANTI-HORSE SERUM (NO. 273) | |
|---|---|---|
| | Before injection | After 48 hours |
| 1:2 | 0 | ++++ |
| 1:5 | 0 | ++++ |
| 1:10 | 0 | +++ |
| 1:100 | 0 | + |
| 1:1000 | 0 | 0 |

TABLE 2

| DILUTION OF HORSE SERUM | PRECIPITATE OBTAINED WITH 0.2 CC. OF ANTI-HORSE SERUM FROM RABBIT (NO. 273) | DILUTION OF SERUM OF RABBIT 291 WHICH PRODUCED A CORRESPONDING PRECIPITATE WITH 0.2 CC. OF THE SAME ANTI-HORSE SERUM FROM RABBIT (NO. 273) |
|---|---|---|
| 1:100 | ++++ | 1:2 |
| 1:1000 | ++++ | 1:5 |
| 1:10000 | ++ | 1:50(?) |
| 1:20000 | + | 1:100 |
| 1:50000 | 0 | 1:1000 |
| 1:100000 | 0 | |

$$\text{Concentration of antigen} = \frac{1}{200}$$

varying from a dilution of 1:100 to a dilution of 1:100,000 the precipitates obtained represent a scale by which the amount of antigen in the blood of rabbit 291 may be roughly estimated.

The foregoing comparison indicates that the blood serum of rabbit 291 forty-eight hours after injection of 1 cc. of horse serum gave the reactions produced by horse serum diluted 200 times. The concentration of precipitiable antigen in the blood serum may be roughly estimated as 1:200.

To determine the period within which antigen remains in the blood serum 1 cc. of donkey serum was injected subcutaneously into a rabbit (no. 289) and on subsequent days the blood was tested for the antigen by means of antidonkey serum from rabbit.

Antigen has been demonstrable in the serum from 7 to 11 days after its introduction into the subcutaneous tissue. Disappearance of antigen has been coincident with appearance of precipitin. After 7 days both antigen and antibody are present in the serum but antigen has diminished before the antibody has become demonstrable.

TABLE 3

*Antigen (donkey) in serum after its injection into normal rabbit*

| | | Precipitate with 0.2 cc. anti-donkey serum from rabbit | | | | | |
|---|---|---|---|---|---|---|---|
| | | Before injection | After 1 day | After 3 days | After 7 days | After 11 days | After 21 days |
| 1 cc. of serum diluted | 1:10 | – | +++ | ++ | + | – | – |
| | 1:100 | – | + | + | – | – | – |
| | 1:1000 | – | – | – | – | – | – |
| Estimated concentration of antigen | | 0 | $\frac{1}{100}$ | $\frac{1}{200}$ | $\frac{1}{1000}$ | 0 | 0 |
| Precipitin titer | | 0 | 0 | 0 | 1:1000 | 1:10000 | 1:1000 |

The fate of donkey serum (1 cc.) introduced into the peritoneal cavity of a rabbit (no. 290) was similar to that of foreign serum given subcutaneously.

Antigen has persisted in the serum during a period from five to nine days in duration and has disappeared when precipitin has appeared.

In these experiments antigen has persisted in the serum during approximately one week. It is possible for the antigen to remain a much longer time.

Rabbit 288 received 1 cc. of horse serum subcutaneously.

Antigen persisted in the serum during 19 days; precipitin developed tardily and in scant strength. It is noteworthy that

this animal with subsequent injections of horse serum failed to develop a high precipitating serum and gave other evidence of deficient immunization.

TABLE 4

*Antigen (donkey) in serum after its injection into normal rabbit*

| | PRECIPITATE WITH 0.2 CC. ANTI-DONKEY SERUM FROM RABBIT | | | | |
|---|---|---|---|---|---|
| | Before injection | After 1 day | After 5 days | After 9 days | After 19 days |
| 1 cc. of serum diluted 1:10 | – | +++ | ++ | – | – |
| 1 cc. of serum diluted 1:100 | – | + | + | – | – |
| 1 cc. of serum diluted 1:1000 | – | – | – | – | – |
| Estimated concentration of antigen | 0 | 1:100 | 1:200 | 0 | 0 |
| Precipitin titer | 0 | 0 | 0 | 1:10000 | 1:20000 |

TABLE 5

*Antigen (horse) in serum after its injection into normal rabbit*

| | PRECIPITATE WITH 0.2 CC. ANTI-HORSE SERUM FROM RABBIT | | | | | | |
|---|---|---|---|---|---|---|---|
| | Before injection | After 1 day | After 2 days | After 3 days | After 5 days | After 7 days | After 9 days |
| 1 cc. of serum diluted 1:10 | – | +++ | ++++ | +++ | +++ | +++ | +++ |
| 1 cc. of serum diluted 1:100 | – | – | ++ | + | + | + | + |
| 1 cc. of serum diluted 1:1000 | – | – | – | – | – | – | – |
| Estimated concentration of antigen | 0 | 1/200 | 1/100 | 1/200 | 1/200 | 1/500 | 1/500 |
| Precipitin titer | 0 | 0 | | | 0 | 0 | 1:1000 |
| | **PRECIPITATE WITH 0.2 CC. ANTI-HORSE SERUM FROM RABBIT** | | | | | | |
| | After 11 days | After 13 days | After 16 days | After 19 days | After 23 days | After 27 days | After 31 days |
| 1 cc. of serum diluted 1:10 | ++ | ++ | ++ | + | – | – | – |
| 1 cc. of serum diluted 1:100 | – | – | – | – | – | – | – |
| 1 cc. of serum diluted 1:1000 | – | – | – | – | – | – | – |
| Estimated concentration of antigen | 1/1000 | 1/5000 | 1/1000 | 1/5000 | 0 | 0 | 0 |
| Precipitin titer | 1:10000 | 1:1000 | 1:10000 | 0 | 0 | 1:100 | 0 |

### ANTIGEN IN THE BLOOD OF IMMUNE ANIMALS

The coexistence of antigen and its specific precipitin in the blood without union to form precipitate is a paradox which has been the subject of much speculation. Some of those who have interested themselves in this subject have reached the conclusion that precipitates are not formed within the body when antigen comes into contact with precipitating antibody. This view suggests that the phenomenon of precipitation has no part in the changes which bring about the destruction or elimination of a protein or protein-like substance introduced into the body. It is assumed that the precipitins formed during the progress of immunization do not combine with the antigen and render it insoluble.

TABLE 6

*Immunization against horse serum*

| | | Test for antigen: precipitate with 0.2 cc. anti-horse rabbit serum | | | |
|---|---|---|---|---|---|
| | | Before injection | After 1 day | After 2 days | After 3 days |
| 1 cc. of serum 273 | 1:10 | 0 | 0 | 0 | 0 |
| | 1:100 | 0 | 0 | 0 | 0 |
| | 1:1000 | 0 | 0 | 0 | 0 |
| Precipitin titer | | 20,000 | 20,000 | | |

*Experiments with horse serum.* After the first injection of an antigen it appears in the blood serum and remains demonstrable during approximately one week or longer but after repeated injections the antigen fails to appear in the blood.

A rabbit (no. 273) received four intramuscular injections of 1 cc. of horse serum. The fifth injection was given into the subcutaneous tissue of the abdomen nine days after the fourth. Tests for antigen and antibody were as shown in table 6.

Antigen failed to appear in the serum and the precipitin titer underwent no change.

The failure of antigen to appear in the blood of a well immunized animal is illustrated by the following experiment.

Rabbit 274 received four intramuscular injections of 1 cc. of horse serum. Twenty-four, forty-eight and seventy-two hours after the fifth injection (1 cc. into the subcutaneous tissue of the abdomen) the blood serum contained no antigen; after the first twenty-four hours the precipitin titer which was 1:20,000 before injection was unchanged. At the sixth injection the quantity given subcutaneously was increased to 2 cc.; no antigen appeared in the blood serum tested after twenty-four and seventy-two hours. The precipitin titer which before injection was 20,000 fell after twenty-four hours to 10,000 but after seventy-two hours was slightly above its former level. At the seventh injection the quantity given was further increased to 4 cc. but no antigen appeared in the serum tested after twenty-four and seventy-two hours. The precipitin titer of the serum which was 50,000 before injection was unchanged after twenty-four hours. At the eighth injection 1 cc. of horse serum was given subcutaneously and the serum was tested in dilutions of 1:5, 1:10, 1:100, 1:1000 with 0.2 cc. anti-horse serum after one, four and twenty-four hours to determine if antigen had entered the blood serum shortly after its administration. No antigen was demonstrable and the only change in precipitin (20,000 before injection) was an insignificant diminution observed 1 hour after administration of serum.

Although an antigen represented by horse serum passes with great rapidity into the blood of a normal animal the serum of a well immunized animal has contained none even though a large quantity (4 cc.) is injected into the subcutaneous tissue. After the injection of a smaller amount none has been found even though tests were made one and four hours after injection.

*Experiments with serum of donkey.* In the following experiments donkey serum was injected into rabbits to determine if it appeared in the blood of an immunized animal.

Rabbit 283 which had received 8 injections of 1 cc. of donkey serum into the muscle or into the subcutaneous tissue of the back was injected subcutaneously with 1 cc. of donkey serum. Before injection there was no antigen demonstrable in the blood serum and the precipitin titer was 50,000. The blood serum tested after twenty-four and after seventy-two hours in dilutions varying from 1:5 to 1:1000 gave no precipitate; the precipitin titer fell to 20,000 at the end of twenty-four hours but had returned to 50,000 after seventy-two hours.

Rabbit 282 which had received six injections of 1 cc. of donkey serum intramuscularly or subcutaneously was injected beneath the skin with 1 cc. of donkey serum. Antigen absent just before injection was not demonstrable in the blood serum after twenty-four hours, after forty-eight hours and after seventy-two hours. The precipitin titer which was 20,000 before injection fell to 1000 but after forty-eight and seventy-two hours had the original level of 20,000. Injections were continued and the animal received 1 cc. of donkey serum subcutaneously fifteen days after the ninth injection. Tests for antigen in the blood serum after the lapse of one hour, of four hours and of twenty-four hours were uniformly negative; the precipitin titer which was 20,000 before injection retained this level after one and four hours but after twenty-four hours had fallen to 10,000.

In animals which had received six injections of donkey serum at intervals of from approximately five to seven days the subcutaneous injection of 1 cc. of donkey serum failed to cause the appearance of this antigen in the blood serum. Tests were negative even though made within the first few hours after administration of the antigen. Nevertheless administration of antigen has caused a well defined but temporary fall in the titer of precipitin in the serum. It seems probable that precipitin has united with antigen in such quantity that its concentration in the serum is perceptibly diminished.

The following experiment is recorded as confirmatory evidence of the great capacity of the immune animal to remove antigen from its body. A preceding experiment has shown that 1 cc. of donkey serum injected into the peritoneal cavity of a normal animal appears in the blood and persists during a period greater than five days and less than nine days. In the tests recorded in table 7 made with serum of an immunized animal (rabbit 284) 1 and 3 cc. of donkey serum caused the appearance of antigen in the serum after twenty-four hours but it had disappeared after seventy-two hours. A peritoneal injection of 6 cc. caused the death of the animal.

Rabbit 284 received six injections of donkey serum (0.5 to 1 cc.) at intervals of about five days. At the seventh injection 1 cc. of donkey serum was injected into the peritoneal cavity. The eighth injection

which was 3 cc. of donkey serum introduced into the peritoneum was eleven days after the seventh. The ninth injection which was made with 6 cc. of donkey serum was seven days after the eighth.

Although there has been transient appearance of antigen in the blood serum of this immunized animal the experiment furnishes conspicuous evidence of increased ability to eliminate antigen from the blood. This power has not been overcome by 3 cc. of donkey serum introduced into the peritoneum but the animal has been overwhelmed by 6 cc. Fall of the precipitin titer is coincident in each instance with injection of antigen.

TABLE 7

*Immunization against donkey serum*

| SEVENTH INJECTION (1 CC. INTO PERITONEAL CAVITY) | TESTS FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. ANTI-HORSE SERUM FROM RABBIT | | | |
|---|---|---|---|---|
| | Before injection | After 1 day | After 3 days | After 11 days |
| 1 cc. of serum 284 1:10 | 0 | + | 0 | 0 |
| 1 cc. of serum 284 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 20,000 | 1,000 | 20,000 | 20,000 |
| **EIGHTH INJECTION (3 CC. INTO PERITONEAL CAVITY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 3 days | After 6 days |
| 1 cc. of serum 284 1:10 | 0 | + | 0 | 0 |
| 1 cc. of serum 284 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 20,000 | 20,000 (Trace) | 20,000 | 20,000 |
| **NINTH INJECTION (6 CC. INTO PERITONEAL CAVITY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 2 days | |
| 1 cc. of serum 284 1:5 | 0 | +++ | Died | |
| 1 cc. of serum 284 1:10 | 0 | +++ | | |
| 1 cc. of serum 284 1:100 | 0 | + | | |
| 1 cc. of serum 284 1:1000 | 0 | 0 | | |
| Precipitin titer | 20,000 | 1,000 | | |

*Experiments with egg white.* Experiments of those who have found that antigen appears in the blood serum of immunized as well as of normal animals have been performed with egg albumin and in the experiments which follow this substance has been employed. In two instances antigen present in the blood serum after the sixth injection failed to appear after the seventh.

TABLE 8

*Immunization against egg white*

| SIXTH INJECTION (1 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. ANTI-EGG SERUM FROM RABBIT | | |
|---|---|---|---|
| | Before injection | After 1 day | After 9 days |
| 1 cc. of serum 271 1:10 | 0 | + | 0 |
| 1 cc. of serum 271 1:100 | 0 | 0 | 0 |
| Precipitin titer | 100,000 | 10,000 | 100,000 |
| **SEVENTH INJECTION (1 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | |
| | Before injection | After 1 day | After 18 days |
| 1 cc. of serum 271 1:10 | 0 | 0 | 0 |
| 1 cc. of serum 271 1:100 | 0 | 0 | 0 |
| Precipitin titer | 100,000 | 10,000 | 50,000 |
| **EIGHTH INJECTION (1 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | |
| | Before injection | After 1 day | After 3 days |
| 1 cc. of serum 271 1:10 | 0 | 0 | 0 |
| 1 cc. of serum 271 1:100 | 0 | 0 | 0 |
| Precipitin titer | 50,000 | 10,000 | 50,000 |

With immunization there has been progressively increased ability to fix or destroy antigen so that it is no longer disseminated throughout the body.

Rabbit 271 which had received two intravenous injections of 2.5 cc. of egg white diluted with an equal quantity of normal salt solution and three intramuscular injections of the same quantity, was injected subcutaneously with 1 cc. of egg white nine days after the fifth injection.

Precipitin tests with the blood serum are recorded in table 8. The seventh injection (0.5 cc. subcutaneously) was made nine days later.

TABLE 9

*Immunization against egg white*

| FOURTH INJECTION (1 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. ANTI-EGG SERUM FROM RABBIT | | |
|---|---|---|---|
| | Before injection | After 1 day | After 9 days |
| 1 cc. of serum 275 1:10 | 0 | + | 0 |
| 1 cc. of serum 275 1:100 | 0 | + | 0 |
| Precipitin titer | 50,000 | 10,000 | 100,000 |

| FIFTH INJECTION (1 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN | | |
|---|---|---|---|
| | Before injection | After 1 day | After 19 days |
| 1 cc. of serum 275 1:10 | 0 | + | 0 |
| 1 cc. of serum 275 1:100 | 0 | + | 0 |
| 1 cc. of serum 275 1:1000 | 0 | 0 | 0 |
| Precipitin titer | 100,000 | 10,000 | 20,000 |

| SIXTH INJECTION (1 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN | | | |
|---|---|---|---|---|
| | Before injection | After 1 day | After 3 days | After 12 days |
| 1 cc. of serum 275 1:10 | 0 | + | 0 | 0 |
| 1 cc. of serum 275 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 20,000 | 10,000 | 20,000 | 20,000 |

| SEVENTH INJECTION (1 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN | | | |
|---|---|---|---|---|
| | Before injection | After 1 day | After 3 days | After 14 days |
| 1 cc. of serum 275 1:5 | 0 | 0 | 0 | — |
| 1 cc. of serum 275 1:10 | 0 | 0 | 0 | — |
| 1 cc. of serum 275 1:100 | 0 | 0 | 0 | — |
| Precipitin titer | 20,000 | 1,000 | 10,000 | 100,000 |

The eighth injection (0.5 cc. subcutaneously) was made seventeen days after the seventh.

Rabbit 275 received into the ear vein 2.5 cc. of egg white diluted with an equal volume of salt solution; after intervals of a week two injections of 1 cc. of egg white were given into the muscle of the back. The fourth injection was 1 cc. given subcutaneously. The results of precipitin tests are recorded in table 9. The fifth injection (0.5 cc. subcutaneously)

TABLE 10

*Immunization against egg white*

| FOURTH INJECTION (0.5 CC. SUBCUTANEOUSLY) | TESTS FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. ANTI-EGG SERUM FROM RABBIT | | | |
|---|---|---|---|---|
| | Before injection | After 1 day | After 3 days | After 5 days |
| 1 cc. of serum 298 1:10 | 0 | + | + | 0 |
| 1 cc. of serum 298 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 10,000 | 10,000 | 10,000 | 20,000 |
| **SIXTH INJECTION (0.5 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 3 days | After 18 days |
| 1 cc. of serum 298 1:2 | – | +++ | 0 | 0 |
| 1 cc. of serum 298 1:10 | 0 | + | 0 | 0 |
| 1 cc. of serum 298 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 20,000 | 1,000 | 1,000 | 1,000 |
| **SEVENTH INJECTION (0.5 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 3 days | |
| 1 cc. of serum 298 1:5 | 0 | ++ | 0 | |
| 1 cc. of serum 298 1:10 | 0 | ++ | 0 | |
| 1 cc. of serum 298 1:100 | 0 | + | 0 | |
| 1 cc. of serum 298 1:1000 | 0 | 0 | 0 | |
| Precipitin titer | 1,000 | 1,000 | 1,000 | |

was made 9 days after the fourth. The sixth injection (0.5 cc. into the subcutaneous tissue) was given nineteen days after the fifth. The seventh injection (0.5 cc. into the subcutaneous tissue) was given twelve days after the sixth.

In these two experiments antigen was present in the serum of the animals immunized against egg albumin after the sixth

injection but failed to make its appearance after the seventh injection. In both instances the precipitin formation was active so that the titer reached a high level (from 30,000 to 100,000). It exhibited a moderate fall after injection of antigen but rapidly recovered its original height.

Rabbit 298 received 1 cc. of egg white subcutaneously, 0.5 cc., eight days later and 1 cc., after an interval of five days. The fourth injection (0.5 cc. subcutaneously) was made fourteen days after the third. The sixth injection (0.5 cc. subcutaneously) was made seven days after the fifth. The seventh injection (0.5 cc. subcutaneously) was made eighteen days after the sixth.

In this experiment antigen has appeared in the serum of the animal immunized against egg white even after seven injections but with the sixth and seventh injections it has been found only after 24 hours whereas after the fourth injection it has still been present after 72 hours. It is noteworthy that antibody formation has been unusually retarded reaching a titer of 20000 only with the sixth injection; in consequence of the injection it has been reduced to 1000 and has never again risen above this level.

*Experiments with crystalline egg albumin.* Crystalline egg albumin was used as an antigen in order to determine if the behavior of a single protein prepared by the method of Hopkins and Pinkus and purified by repeated recrystallization differed from the complex mixtures represented by mammalian serum and by egg white. In the following experiment tests for antigen and antibody were made throughout the course of immunization against crystalline egg albumin.

Rabbit 420 received at intervals of five days 0.5 cc. of a 5 per cent solution of crystalline egg albumin injected into the subcutaneous tissue. The result of tests made after the first injection were as shown in table 11.

The behavior of crystalline egg albumin has not been identical with that of more complex antigens such as blood serum and egg white. (a) Crystaline egg albumin disappears from the blood of both the normal and immune animal more rapidly than antigen of serum or of egg white. (b) Nevertheless with im-

TABLE 11

*Immunization against crystalline egg albumin*

| FIRST INJECTION (0.5 CC. SUBCUTANEOUSLY) | TEST FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. OF ANTI-OVALBUMIN SERUM FROM RABBIT | | | |
|---|---|---|---|---|
| | Before injection | After 1 day | After 2 days | After 5 days |
| 1 cc. of serum 420, 1:10 | 0 | + | + | 0 |
| 1 cc. of serum 420, 1:50 | 0 | + | + | 0 |
| 1 cc. of serum 420, 1:100 | – | + | 0 | 0 |
| Precipitin titer | 0 | 0 | 0 | 0 |
| **SECOND INJECTION (0.5 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 2 days | After 5 days |
| 1 cc. of serum 420, 1:10 | 0 | + | 0 | 0 |
| 1 cc. of serum 420, 1:50 | 0 | + | 0 | 0 |
| 1 cc. of serum 420, 1:100 | 0 | 0 | 0 | 0 |
| Precipitin titer | 0 | 0 | 0 | 1,000 |
| **THIRD INJECTION (0.5 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 2 days | After 5 days |
| 1 cc. of serum 420, diluted as above | 0 | 0 | 0 | 0 |
| Precipitin titer | 1,000 | 0 | 0 | 20,000 |
| **FOURTH INJECTION (1 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 2 days | After 5 days |
| Test for antigen | 0 | 0 | 0 | – |
| Precipitin titer | 20,000 | 0 | 20,000 | 50,000 |
| **FIFTH INJECTION (3 CC. SUBCUTANEOUSLY)** | **TESTS FOR ANTIGEN** | | | |
| | Before injection | After 1 day | After 2 days | After 11 days |
| 1 cc. of serum 431, 1:2 | – | +++ | 0 | 0 |
| 1 cc. of serum 431, 1:5 | – | +++ | 0 | 0 |
| 1 cc. of serum 431, 1:10 | – | ++ | 0 | 0 |
| 1 cc. of serum 431, 1:100 | – | + | 0 | 0 |
| 1 cc. of serum 431, 1:1000 | – | 0 | 0 | 0 |
| Precipitin titer | 50,000 | 0 | 0 | 100,000 |

TABLE 11—*Continued*

| SIXTH INJECTION (3 CC. SUBCUTANEOUSLY) | | TESTS FOR ANTIGEN | | | |
|---|---|---|---|---|---|
| | | Before injection | After 1 day | After 2 days | After 4 days |
| 1 cc. of serum 431 | 1:5 | 0 | + | 0 | 0 |
| | 1:10 | 0 | + | 0 | 0 |
| | 1:100 | 0 | + | 0 | 0 |
| | 1:1000 | 0 | 0 | 0 | 0 |
| Precipitin titer | | 100,000 | 0 | 20,000 | 100,000 |

munization there is a progressive increase in the ability of the body to rid itself of the antigen so that it enters the blood with increasing difficulty and, should it enter the blood, quickly disappears. After the first injection antigen failed to appear in the blood, until the amount injected was increased to 3 cc. and the effect of the second injection of 3 cc. was less than that of the first. (c) Injection of antigen has repeatedly caused complete disappearance of antibody so that precipitin present in high titer before injection has completely disappeared; its disappearance is temporary. (d) Antigen and antibody have never been demonstrated in the same serum.

The following experiments were performed to determine if injection of crystalline egg albumin in considerable quantity, namely, 3 cc. of a 5 per cent solution into an immunized animal was uniformly followed by disappearance of precipitin from the circulating blood.

Rabbit 433 was immunized against crystalline egg albumin by daily subcutaneous injection of 0.5 cc. of a 5 per cent solution during sixteen days. Nine days later 3 cc. of the same solution were injected into the subcutaneous tissue of the back. Precipitin tests were as shown in table 12.

Following the injection of antigen precipitin disappeared completely from the circulating blood at the time when antigen was demonstrable. Precipitin returned slowly so that two days after injection no precipitate was obtainable with the lower dilutions of serum (from 1:10 to 1:1000) whereas with higher

dilutions (1:10,000 to 1:50,000) traces of precipitate were formed. After 4 days precipitin in the serum exceeded its original strength.

TABLE 12

*Immunization against crystalline egg albumin*

| | | TEST FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. OF ANTI-OVALBUMIN SERUM FROM RABBIT | | | |
|---|---|---|---|---|---|
| | | Before injection | After 1 day | After 2 days | After 4 days |
| 1 cc. of serum 433 | 1:5 | 0 | ++ | 0 | 0 |
| | 1:10 | 0 | ++ | 0 | 0 |
| | 1:100 | 0 | 0 | 0 | 0 |
| | 1:1000 | 0 | 0 | 0 | 0 |
| Precipitin titer | | 50,000 | 0 | 50,000 (Trace) | 100,000 |

Rabbit 418 received twelve subcutaneous injections of 0.5 cc. of a 5 per cent solution of crystalline egg albumin at intervals of from five to ten days. Twelve days after the last injection 3 cc. of the same solution were injected into the subcutaneous tissue. Precipitin tests were as shown in table 13.

TABLE 13

*Immunization against crystalline egg albumin*

| | | TEST FOR ANTIGEN: PRECIPITATE WITH 0.2 CC. OF ANTI-OVALBUMIN SERUM FROM RABBIT | | | |
|---|---|---|---|---|---|
| | | Before injection | After 1 day | After 2 days | After 4 days |
| 1 cc. of serum 418 | 1:5 | 0 | + | 0 | 0 |
| | 1:10 | 0 | + | 0 | 0 |
| | 1:100 | 0 | 0 | 0 | 0 |
| | 1:1000 | 0 | 0 | 0 | 0 |
| Precipitin titer | | 100,000 | 0 | 0 | 50,000 |

The experiment confirms those previously cited. Following the injection of a considerable quantity of crystalline egg albumin this antigen has appeared in the circulating blood and precipitin has coincidently disappeared. Reappearance of precipitin has occurred even more slowly than in the previous experiment so

that it remains absent after two days and though present in considerable strength has not completely returned to its original level after four days. It is noteworthy that injection of serum (3 cc. to 6 cc.) or of egg white (1 cc.) has uniformly failed to remove antibodies from the circulating blood whereas injection of from 1 to 3 cc. of single protein as antigen, namely purified crystalline egg albumin, has in every experiment removed all precipitins from the blood of rabbits immunized against this substance.

It is well known that the supernatant fluid over a precipitate formed by the mixture of an antigen such as blood serum or egg white and its precipitin may contain free antigen and antibody which have failed to unite to form precipitate. In a preceding paper evidence was presented to show that serum and egg white are complex mixtures of antigens which produce a corresponding variety of precipitins whereas crystalline egg albumin purified by repeated crystallization is a relatively simple substance and represents a single antigen mixed with scant traces of other protein. This view furnishes a satisfactory explanation of the phenomena which have been observed in the living animal. Injection of horse serum, of donkey serum or of egg white into an animal immunized against one or other of these substances does not remove all precipitin from the circulating serum. When the complex mixture of antigens contained in one or other of these substances is injected into an animal the strength of various antibodies which make their appearance in the circulating blood of the immunized animal doubtless bears no direct relation to the relative strength of various antigens in serum in one instance or in egg white in the other. Certain antibodies are present in such excess that they are not completely precipitated when the substance employed is again injected into the immune animal, they persist in the serum and react with dilutions of serum or of egg white which contain all of the antigens against which the animal is immunized. Immediately after injection antigen may appear in the serum and is demonstrable by addition of immune serum from another animal, which contains all of the antibodies formed in response to the substance employed for

immunization. Antigen which is demonstrable in the blood then fails to form a precipitate with the antibodies present in the same serum because it does not stand in relation of precipitinogen to that precipitin which persists in the serum. When a simple antigen such as crystalline egg albumin is employed its injection causes complete disappearance of precipitin from the circulating blood for all of the precipitin corresponds to the precipitinogen injected. Following injection of purified crystalline egg albumin antigen appears in the blood serum but antigen and antibody have not been demonstrable simultaneously in the circulating blood.

### CONCLUSIONS

Antigens such as horse serum or egg white injected into a normal animal are demonstrable in the blood during a period from approximately seven to nine days after injection; antigen disappears shortly after the appearance of precipitin at a time when the concentration of precipitins is rapidly increasing.

In exceptional instances antigen persists during a much longer period for example during nineteen days and in these animals formation of precipitins proceed slowly and their concentration in the serum remains low.

With the progress of immunization injected antigen exhibits a decreasing tendency to find its way into the circulating blood so that in a well immunized animal horse serum or egg white injected into the subcutaneous tissue is not demonstrable in the blood serum even if tests are made at intervals from one to twenty-four hours after injection.

Complex antigens such as horse serum or egg white injected into an immunized animal cause a diminution of the concentration of precipitins but precipitins do not disappear completely and in the early stages of immunization both antigen and antibody may be demonstrable in the same serum.

A simple antigen, namely, crystalline egg albumin, purified by repeated crystallization, injected into an animal immunized against this substance may cause temporary but complete disappearance of precipitin and though antigen may appear in the

serum in no instance has this antigen and its precipitin been simultaneously demonstrable in the serum.

The foregoing observations upon living animals, like the coexistence *in vitro* of antigen and antibody which fail to form a precipitate, are explained by the presence of multiple antigens in mammalian serum or in egg white and a corresponding multiplicity of antibodies in immune sera. Crystalline egg albumin carefully prepared by the usual methods is a relatively pure antigen and bring about the formation of a single precipitin. The behavior of precipitins in the living body is similar to that in the test tube and indicates that they precipitate foreign protein introduced into the immune animal.

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# PHYSIOLOGICAL ADAPTATIONS OF FIXED-TISSUES IN ANAPHYLAXIS AND IMMUNITY

## I. REACTIONS OF THE ISOLATED RABBIT HEART TO COBRA VENOM[1]

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Our knowledge of the immuno-physiological adaptations of fixed-tissues is at present limited to an elementary conception of the altered excitability of non-striated muscle as a result of protein sensitization. We therefore began in 1916 a study of the physiological adaptations of other fixed tissues. We selected the isolated rabbit heart as the initial organ to be tested, and found an increased resistance of the cardiac tissues to the toxic action of goat serum, as a result of sensitization and immunization (1, 2).

The cardiac reactions we then obtained, however, were inconstant. On resuming this line of work we have therefore sought substances of immunological importance giving constant, sharply defined reactions with the excised heart. Cobra venom is a substance well suited for such tests.

### I. REACTIONS OF THE NORMAL RABBIT HEART TO COBRA VENOM

The technique employed in testing the excised mammalian heart is the simplified technique described by Gunn (3). The arrangement of the apparatus is shown in figure 1. Rabbit hearts isolated by this method and perfused with well aerated Locke's solution, beat regularly and strongly for nearly two hours.

[1] Presented before the American Association of Immunologists, Washington, D. C., May 1, 1922.

If cobra venom is added to the Locke's solution, there are produced a number of variations from the normal picture. Certain of these variations are inconstant, and cannot be taken as reliable indices of venom action. Among these minor reactions are: (a) *an initial tachycardia,* (b) *a terminal tachycardia,* and (c) *heart block.* These reactions are usually observed when cobra venom is tested in dilute solutions, but are often absent with stronger venom solutions. The constant reactions are:

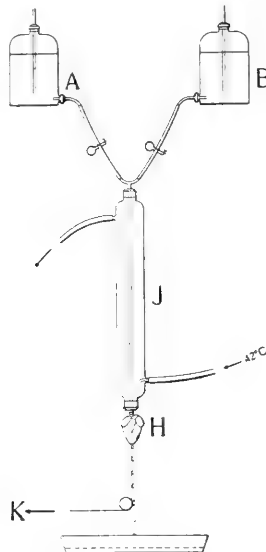


FIG. 1. PERFUSION APPARATUS FOR ISOLATED MAMMALIAN HEARTS

*A* = Constant pressure bottle containing well aerated Locke's solution.
*B* = Constant pressure bottle containing solution to be tested.
*J* = Constant temperature water jacket. A continuous flow of water at 42°C. is maintained through this jacket by means of a centrifugal pump.
*H* = Isolated heart.
*K* = Thread to recording apparatus.

(d) *Decreased myocardial contractions,* shown by decreased height of the kymograph tracing (fig. 2). This reaction usually begins in from one to ten minutes after the addition of the venom solution. It is occasionally preceded by a preliminary period of increased contractions. Once established, however, the phenome-

non is progressive, ending in complete cessation of recordable movements. Visible contractions usually cease first in the left ventricle, somewhat later in the right ventricle and, after a delay of several minutes, in the auricles.

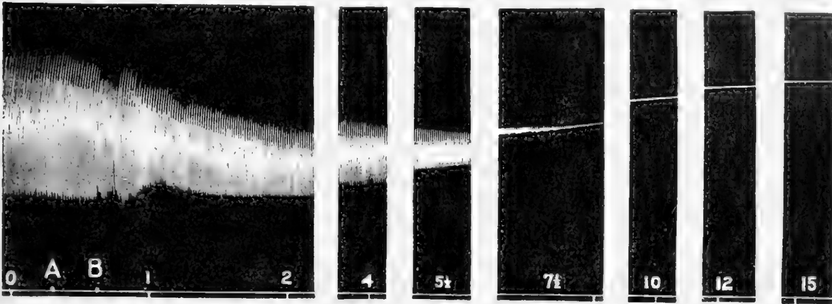


FIG. 2. REACTIONS OF ISOLATED RABBIT HEART TO COBRA VENOM

Normal heart; 1:100,000 cobra venom; perfusion pressure 60 mm. Hg., time in minutes.

*A* = Time of changing perfusion clamps.

*B* = Estimated time of entrance of venom solution into coronary artery.

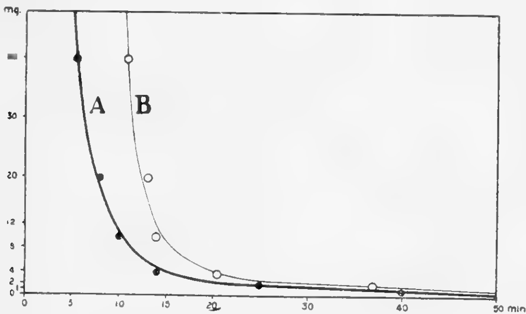


FIG. 3. RELATION BETWEEN VENOM CONCENTRATION AND REACTION TIMES

Composite data from twenty-four normal hearts. Concentration shown as milligrams of cobra venom per litre of perfusion fluid; e.g., 1 mgm. = 1:1,000,000 C. V.

Curve A = Time of cessation of recordable contractions.

Curve B = Time of establishment of final maximum tone.

The length of time it takes a venom solution to bring the heart to complete standstill, varies with the strength of the venom solution tested (fig. 3). The time may be as short as three

minutes with strong venom solutions, increasing to from thirty-five to fifty minutes with dilute venom solution. The time relationship is sufficiently constant to serve as a titration index for cobra venom.

(e) *Increased myocardial tone,* shown by a rise in the base line of the kymograph tracing (fig. 2). This reaction usually begins in from three to fifteen minutes after the addition of the venom solution. It is occasionally preceded by a preliminary relaxation of tone. Once established, however, this phenomenon also is progressive, leading to a final maximum tone of the myocardium.

The length of time it takes a venom solution to bring the heart to this final maximum tone varies with the concentration of the venom solution tested (fig. 3). The time may be as short as five minutes with strong venom solution, increasing to from forty to sixty minutes with dilute venom solutions. This time relationship also is sufficiently constant to serve as a titration index for cobra venom.

## II. SOURCES OF ERROR

In testing the reactions of the isolated rabbit heart to cobra venom we have come to recognize certain possible sources of error. Among these are:

(a) *Seasonal variations in cardiac resistance.* The isolated rabbit heart has a maximum cobra venom resistance during the summer months, and a distinct cobra venom hypersusceptibility during the winter months. Thus the heart of a normal autumn rabbit requires a dilution of 1:200,000 cobra venom in order to bring it to a complete standstill in twenty minutes, while the heart of a normal winter rabbit is brought to complete standstill in the same period of time by a venom dilution as great as 1:750,000.

(b) *Hypersensitiveness from intercurrent infections.* A cobra venom hypersusceptibility is produced by intercurrent infections. Thus in two of our parallel tests, the heart of a normal rabbit resisted inactivation by 1:500,000 C.V. for thirty-five minutes, while the heart of a "snuffles" rabbit was inactivated by the same venom solution in fifteen minutes.

(c) *Hypersensitiveness from previous toxic injury.* Equally marked venom hypersusceptibility is produced by the previous injection of toxic agents. A subcutaneous injection of peptone, for example, increases the cobra venom susceptibility. This hypersusceptibility can be detected as early as twenty-four hours after the peptone injection. It usually disappears within two weeks. This phenomenon was recently described by Heymans (4) as a non-specific anaphylaxis.

(d) *Non-agreement of duplicates.* Care being taken to exclude error from the above sources, it was found that duplicate normal controls differ within fairly narrow limits. Differences in inactivation time rarely exceed two minutes; differences in time of maximum final tone rarely exceed five minutes. Variations within these limits, therefore, are without immunological significance.

### III. METHOD OF SENSITIZATION AND IMMUNIZATION

The cobra venom M.L.D. for medium sized rabbits is approximately 1 mgm. Injected intravenously, 1 mgm. usually kills within twenty minutes; injected subcutaneously, within twelve hours.

Rabbits were injected subcutaneously with an initial dose of 0.1 mgm. cobra venom. The dose was repeated at five day intervals, and gradually increased to a maximum dose of 1 mgm. This maximum was usually reached by the tenth injection. Subsequent injections were usually made at seven day intervals.

(a) *Hypersensitive stage.* About 25 per cent of the rabbits died about the time of the fourth to sixth injection. Rabbits receiving from three to five injections were therefore taken to represent the hypersensitive stage.

(b) *Immune stage.* After ten injections a distinct immunity is demonstrable, rabbits at this stage resisting subcutaneous injections of 20 mgm. cobra venom. This resistance rises to over 80 mgm. by the twentieth injection. Rabbits receiving from ten to twenty venom injections were therefore taken to represent the immune stage.

## IV. MYOCARDIAL ADAPTATIONS

(a) *Transient hypersusceptibility.* A distinct cobra venom hypersusceptibility is demonstrable in the isolated rabbit hearts for at least a week following a subcutaneous venom injection (4). Thus, in two of our parallel tests, the heart of a rabbit, removed three days after a single subcutaneous injection of cobra venom, was inactivated by 1:750,000 C.V. in *8* minutes and thrown into final contraction in *17* minutes, as contrasted with *17* minutes and *28* minutes respectively for its normal control.

This hypersusceptibility usually disappears within ten days. We believe this hypersusceptibility cannot be looked upon as a true anaphylactic phenomenon, but must be regarded as a transient pseudoanaphylaxis due to myocardial injury.

(b) *Permanent adaptations.* In order to avoid error from this transient hypersensitiveness, a convalescent period of at least two weeks was allowed to elapse between the final venom injection and the cardiac test. Hearts tested at this later period have been divided into two groups:

(i) *Hypersensitive stages.* The hearts of six rabbits were tested during the hypersensitive stage. The venom dilutions used in making these tests varied from 1:100,000 to 1:750,000. Three of these hearts gave reactions identical with their normal controls, one heart showed a slight apparent hypersensitiveness, and two hearts a slight apparent immunity. The variation from the normal control, however, was in no case greater than variations occasionally noted between duplicate normal hearts.

The average or composite picture of these six hearts shows a heart inactivated in *14* minutes, and thrown into final contraction in *21.5* minutes, as compared with *13.5* minutes and *20* minutes respectively for the composite normal control.

(ii) *Immune stage.* The hearts of six rabbits were tested in the immune stage. The venom dilutions used in making these tests varied from 1:200,000 to 1:1,000,000. Two of these hearts gave reactions identical with their normal controls, two showed a slight apparent hypersensitiveness, and two a slight apparent immunity. The variation from the normal control, however,

was in no case greater than variations often seen between duplicate normal hearts.

The average or composite picture of these six hearts shows a heart inactivated in *18.2* minutes and thrown into final contraction in *23* minutes, as compared with *19* minutes and *20.5* minutes respectively for the composite normal control.

(iii) *Composite picture.* The composite picture of the twelve hearts tested shows a heart inactivated in *16.1* minutes and thrown into final contraction in *22.3* minutes, as compared with *16.2* minutes and *20.3* minutes respectively for the composite normal control.

## V. CONCLUSIONS

Within the limits of the experimental error, and with the technique employed, there is therefore no demonstrable permanent change in myocardial resistance to cobra venom, either during the hypersensitive stage or during the stage of immunity. The cardiac tissues therefore apparently play a purely passive rôle in immunological adaptation to cobra venom.

## VI. SUMMARY

1. Hearts of rabbits sensitized or immunized to cobra venom are identical with normal rabbit hearts in their resistence to cobra venom.

2. The cardiac tissues therefore apparently play a purely passive rôle in immunological processes against cobra venom.

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# STUDY OF MICROBIC-TISSUE AFFINITY BY PERFUSION METHODS[1]

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In 1916 we began a study of the laws governing the topographical distribution of microörganisms in the animal body by the application of perfusion methods to isolated organs and tissues. Our initial tests were with pneumococci and rabbits. On resuming this line of work we have sought first of all to determine whether or not the laws governing the distribution of penumococci in rabbits hold for other microörganisms and for other animal species. The present report is based on perfusion experiments with dogs.

*Technique.* To make the perfusion tests, an afferent cannula is tied in the main artery supplying the organ or group of tissues to be tested, and an efferent canula in the main vein coming from these parts. The collateral circulation is ligated. The part is washed free from blood by a preliminary prefusion with Ringer's solution, and then repeatedly perfused with Ringer's solution containing a known number of microörganisms per cubic centimeter. Samples of the perfusion fluid are taken at the end of each passage, and the number of residual microörganisms in each sample determined by plating methods. The method of course is limited to the study of microörganisms not killed or agglutinated by serum, and to microörganisms that can be obtained in suspensions free from clumps.

*Interpretation of results.* The microbic-affinity of an organ or group of tissues may be expressed as the average percentage of microörganisms deposited in these tissues per passage of the

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perfusion fluid. For example, if the perfusion fluid originally contained 10,000,000 microörganisms per cubic centimeter, and this number is reduced to 8,000,000 by the end of the first passage, to 6,500,000 by the end of the second passage, and to 5,000,000 by the end of the third passage, the microbic affinity of the tissues tested can be expressed as approximately 20 per cent. We have arbitrarily selected the residual counts at the end of the third and fifth passages as the basis for this calculation. Unless otherwise stated, percentages hereinafter recorded are based on this arbitrary standard.

### I. LAWS GOVERNING MICROBIC-TISSUE AFFINITY

From data thus far obtained, it appears that the microbic affinities of canine tissues are governed by the following laws:

1. *The microbic-tissue affinity varies with the tissue tested.* Data showing the relative affinities of certain canine tissues for *S. aureus* are given in figure 1. From these data it appears that the tissues showing the lowest staphylococcus affinity are the brain and adjacent portions of the meninges. Fully 92 per cent of the staphylococci remain in the perfusion fluid, after twelve passages through these tissues, giving a calculated staphylococcus affinity less than 0.5 per cent.

Most of the tissues, however, show a staphylococcus affinity, varying from 4 per cent (isolated intestine) to 6 per cent (isolated lungs). Two tissues show an exceptionally high staphylococcus affinity: the spleen with a staphylococcus affinity of 60 per cent, and the liver with a staphylococcus affinity of 80 per cent.

2. *The microbic-tissue affinity varies with the microörganism tested.* Data showing the relative hepatic affinities for certain typical microörganisms are given in part I, figure 2. The microörganisms thus far tested may be divided into three groups:

Group I. Microörganisms for which the canine liver has a very high affinity. The hepatic affinity for this group of microörganisms varies from 40 per cent (*B. coli*) to 80 per cent (*S. aureus*).

Group II. Microörganisms for which the canine liver has a relative low affinity. The hepatic affinity for this group varies from 10 per cent (*B. bisepticus*) to 25 per cent (*B. anthracis*).

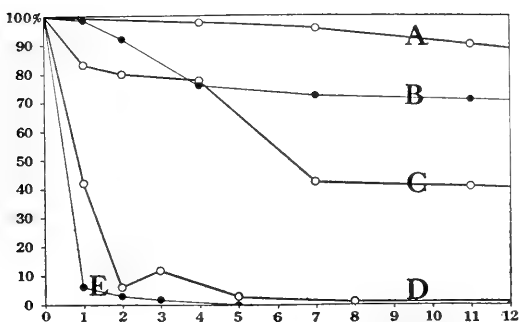


FIG. 1. STAPHYLOCOCCUS AFFINITIES OF DIFFERENT CANINE TISSUES

Perfusions with *S. aureus*, eighteen hours cultures in nutrient broth containing 20 per cent dog serum. The cultures consisted mainly of single cocci with occasional diplocci. No clumps. Perfusion fluids contained from 1,000,000 to 5,000,000 microörganisms per cubic centimeter. Plate counts recorded as percentage s of initial counts.

A = Perfusion of brain and adjacent tissues. Calculated microbic affinity = 0.5 per cent.

B = Perfusion of isolated loop of intestine. Calculated microbic affinity = 4 per cent.

C = Perfusion of lungs. Calculated microbic affinity = 6 per cent.

D = Perfusion of spleen. Calculated microbic affinity = 60 per cent.

E = Perfusion of liver. Calculated microbic affinity = 80 per cent.

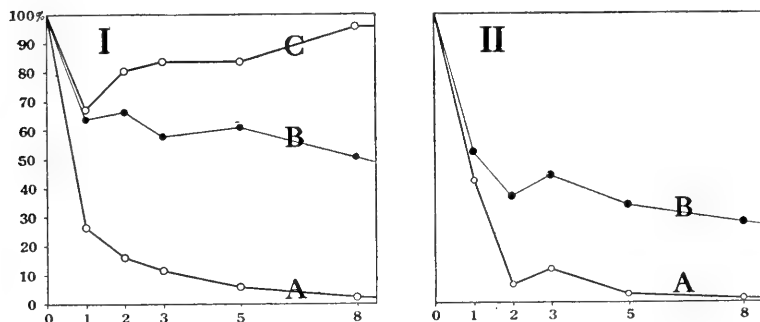


FIG. 2. TISSUE AFFINITIES FOR DIFFERENT MICROÖRGANISMS

*I. Perfusion of normal liver.*

A = Composite picture of *S. aureus* and *B. coli*. Calculated microbic affinity = 60 per cent.

B = Composite picture of *B. anthracis* and *B. bisepticus*. Calculated microbic affinity = 15 per cent.

C = Composite picture of three perfusions with *B. lactis aerogenes*. Calculated microbic affinity = 4 per cent.

*II. Perfusion of normal spleen.*

A = *S. aureus*. Calculated microbic affinity = 60 per cent.

B = *B. lactis aerogenes*. Calculated microbic affinity = 20 per cent.

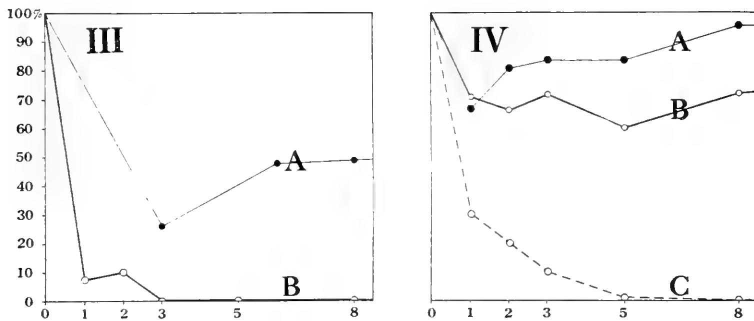


FIG. 3. EFFECTS OF IMMUNIZATION ON MICROBIC-HEPATIC AFFINITY

*III. Perfusions with B. anthracis.*

A = Normal liver (control). Calculated microbic affinity = 20 per cent.

B = Immune liver. Calculated microbic affinity = 80 per cent.

*IV. Perfusions with B. lactis aerogenes.*

A = Normal liver (control). Calculated microbic affinity = 4 per cent.

B = Immune liver. Calculated microbic affinity = 10 per cent.

C = Immune liver with 7 per cent immune serum added to the perfusion fluid. Calculated microbic affinity = 60 per cent.

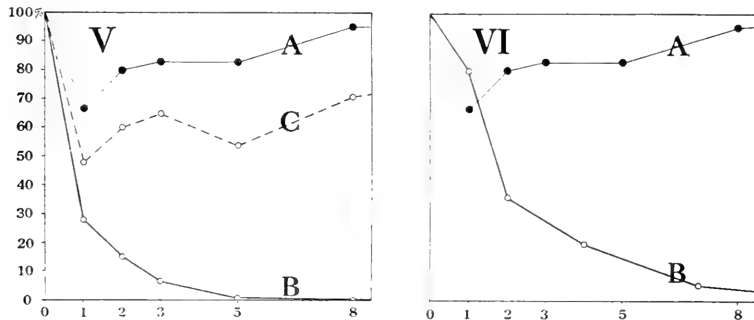


FIG. 4. SEROLOGICAL ANALYSIS

*V. Perfusions of normal liver with B. lactis aerogenes.*

A = Serum free perfusion (control). Calculated microbic affinity = 4 per cent.

B = 7 per cent immune serum added to the perfusion fluid. Calculated microbic affinity = 60 per cent.

C = 7 per cent heated immune serum added to the perfusion fluid. Calculated microbic affinity = 15 per cent.

*VI. Perfusions of normal liver with sensitized microörganisms.*

A = Perfusion with non-sensitized *B. lactis aerogenes* (control). Calculated microbic affinity = 4 per cent.

B = Perfusion with sensitized *B. lactis aerogenes*. Calculated microbic affinity = 40 per cent.

Group III. Microörganisms for which the canine liver has little or no affinity. A typical member of this group is *B. lactis aerogenes.* This microörganism is deposited in the liver in considerable numbers during the first passage of the perfusion fluid, but is washed out from the liver practically quantitavely on subsequent passages of the perfusion fluid. The calculated affinity based on the residual counts at the end of the third and fifth passages is less than 4 per cent.

This grouping of microörganisms with reference to their hepatic affinities gives a grouping that also holds for the spleen (part II, fig. 2.)

*3. The microbic-hepatic affinity is increased by immunization.* Data showing such increases are given in figure 3. The data show the *B. anthracis*-hepatic affinity increased from 25 per cent to 80 per cent, and the *B. lactis aerogenes*-hepatic affinity from 4 per cent to 10 per cent as a result of immunization.

*4. The microbic affinity of the immune liver is further increased by the addition of immune serum in the perfusion fluid.* Data showing such an increase are given in part IV, figure 3. The *B. lactis aerogenes*-hepatic affinity is here increased from 10 per cent to 60 per cent by the addition of 7 per cent immune serum to the perfusion fluid.

*5. A microbic-hepatic affinity practically identical with that of the immune liver is conferred upon a normal liver by the addition of immune serum in the perfusion fluid.* Data showing such passive microbic-hepatic affinity are given in part V, figure 4. Here the addition of 7 per cent immune serum in the perfusion fluid increases the *B. lactis aerogenes* affinity of the normal liver from 4 per cent to 60 per cent.

*6. The serum component responsible for this passive microbic-hepatic affinity is thermolabile.* Heating the immune serum to 60°C. for thirty minutes reduces the passive *B. lactis aerogenes*-hepatic affinity from 60 per cent to 15 per cent (part V, fig. 4).

*7. The serum component increases the microbic-hepatic affinity by acting upon the microörganisms.* Carefully washed, sensitized *B. lactis aerogenes* in serum free suspensions show a calculated hepatic affinity of 40 per cent as contrasted with the 4 per cent

affinity of non-senstitized *B. lactis aerogenes* (part VI, fig. 4). The serum component, therefore, can be conveniently classified as an endothelial opsonin.

*8. The endothelial opsonin is also operative with the spleen. So far as tested however it is inoperative with the other extra-hepatic tissues.* Thus, in a serum free perfusion of canine lungs with *B. anthacis*, 82 per cent of the microörganisms remained in the perfusion fluid at the end of the twelfth passage. A parallel perfusion with 7 per cent immune serum added to the perfusion fluid, gave a residual count of 80 per cent. (The bone marrow has not yet been tested.)

## II. TOPOGRAPHICAL DISTRIBUTION OF MICROÖRGANISMS IN THE ANIMAL BODY

On intravenous injection, therefore, the topographical distribution of microörganisms in canine tissues is apparently governed by the amount of specified endothelial opsonin present in the circulating blood. With different amounts of specific opsonin, the microbic distribution would be approximately as follows:

*1. In the absence of specific endothelial opsonin:* This condition is illustrated by injection of *B. lactis aerogenes* in normal dogs. Here the topographical distribution is governed by a 4 per cent microbic-hepatic affinity, a 20 per cent microbic-splenic affinity, and a microbic affinity varying from 4 per cent to 6 per cent for the remaining tissues, the exception being the 0.5 per cent microbic affinity of the central nervous system. The microorganisms therefore would tend to be fairly evenly distributed throughout the body, with a relatively small deposit in the central nervous system and relatively large deposit in the spleen. On account of the small size of the spleen, however, the splenic localization would not materially reduce the number of microorganisms deposited in other tissues.

*2. In the presence of large amounts of specific endothelial opsonin:* This condition is illustrated by *S. aureus* injections in normal dogs (normal endothelial opsonins) and by *B. lactis aerogenes* injections in immune dogs (immune endothelial opsonins). Here the topographical distribution is governed by an 80 per cent

microbic-hepatic affinity, and a 60 per cent microbic-splenic affinity; the remaining extra-hepatic affinities being the same as in the absence of specific opsonin. On account of its large size and abundant blood supply, the liver would be the dominant factor in this distribution, the majority of the microörganisms being deposited in this organ. The remaining microörganisms would show a relatively large splenic deposit and a distribution throughout the remaining extra-hepatic tissues in approximately the same relative numbers as in the absence of specific opsonin.

*3. In the presence of small amounts of specific endothelial opsonin.* This condition is illustrated by *B. anthracis* and *B. bisepticus* injections in normal dogs. Here the topographical distribution would be intermediate between (a) and (b).

## III. SUMMARY

1. The microbic-tissue affinity of an organ or group of tissues may be expressed as the average percentage of microörganisms deposited in these tissues per passage of a perfusion fluid.

2. The microbic-tissue affinity varies with the organ or group of tissues tested. Thus, the central nervous system shows a microbic affinity of 0.5 per cent, the spleen a microbic affinity varying from 20 per cent to 60 per cent, and the liver a microbic affinity varying from 4 per cent to 80 per cent, depending upon the microörganisms tested. The remaining extra-hepatic tissues show affinities varying from 4 per cent to 6 per cent.

3. The microbic-hepatic affinity is increased by immunization. The immune liver shows an affinity of at least 80 per cent for all microörganisms thus far tested.

4. This increased hepatic affinity is due to the action of specific endothelial opsonins.

5. The endothelial opsonins also increase the microbic-splenic affinity. So far as tested, however, they are inoperative with the other extra-hepatic tissues.

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# THE PROPHYLACTIC ACTION OF ATROPINE SULPHATE UPON THE ANAPHYLACTIC AND ALLERGIC REACTIONS OF THE EXCISED UTERUS OF VIRGIN GUINEA-PIGS[1]

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The "cellular theory" of anaphylaxis seems at the present time to offer the most tenable conception of this phenomenon. That the reaction occurs peripheral to the central nervous system has been well established. Coca (1) and Wells (2) have thoroughly reviewed the literature bearing on the cellular and humoral theories of anaphylaxis and point out that the experimental evidence supports the contention that humoral reactions play no important rôle in anaphylaxis. The experiments on isolated smooth muscle as carried on by Schultz (3) and later by Dale (4) and Weil (5) have supplied valuable data in support of the "cellular theory."

Since the reaction is cellular and peripheral it must act upon the smooth muscle, the nerve or the neuromuscular connections. Auer's (6) experiments in which he demonstrated that the bronchial constriction appeared alike in both lungs of an animal when the vagus nerve on one side had been sectioned and allowed to degenerate seem to exclude the peripheral nerve as a factor in the response. There was, of course, no assurance that the neuromuscular connection had degenerated. Whether the anaphylactic reaction is a result of stimulation of the muscle directly or on the neuromuscular mechanism seems to us to be an open question. Auer (7) and others have demonstrated that atropine

[1] Part of the expense incurred in this research was covered by a grant of the Research Committee of the University of Kansas.

has a marked prophylactic action by preventing the constriction of the bronchial musculature in sensitized guinea-pigs when treated with a fatal dose of the antigen. It has not been shown that the atropine action was entirely on the smooth muscle, rather than on the nerve-endings unless it is assumed that the nerve-endings are degenerated.

So far as we know there is no report in the literature showing the action of atropine on the anaphylctic reaction of smooth muscle *in vitro* where uterine strips are used. Dale (8) has shown that this reaction is suppressed by hypertonic salt solutions.

The work reported in the following pages was undertaken to determine, if possible, whether the anaphylactic reaction as exhibited by uterine muscle of the guinea-pig was confined entirely to the muscle itself. It was hoped that evidence might be obtained that would contribute towards the answer of the following questions.

1. The effect of varying doses of atropine upon the activity of normal and sensitized uterine strips suspended in oxygenated Tyrode's Solution under conditions to be described later.
2. The influence of atropine upon the so-called toxic reactions produced by native proteins when applied to the uteri of normal guinea-pigs.
3. The influence of varying doses of atropine upon the specific response of uterine horns from sensitized guinea-pigs.

## EXPERIMENTAL METHODS

Uterine horns from virgin guinea-pigs weighing from 150 to 450 grams were used in all the experiments. The animals were killed and the uterine horns were quickly removed with the least possible disturbance. Both horns of the uterus were used in all cases. The horns were attached by ligatures to the hook of a muscle warmer and a light heart lever the arms of which had a ratio of 2:1 so that the records on the drums were double the actual contraction. The lever was so weighted that the pull of the muscle was against 0.5 gram except in a few experiments where the horns were very slender when the weight was reduced.

The muscle strips were immersed in 25 cc. Tyrode's solution in the glass tube of the muscle warmer. A steady stream of oxygen was allowed to bubble through the solution. A siphon tube was connected to the muscle warmer so that the Tyrode's solution could be quickly changed without disturbance to the muscle. A uniform temperature of 38°C. was maintained in a solution surrounding the muscle warmer.

Both the left and right horns of the uteri were used, care being taken to perform the tests in the opposite order in different experiments, so as to exclude any possible difference due to keeping one horn longer than the other out of the solution. The Tyrode's solution was prepared according to the ordinary formula as given by Sollman (9) except that one-half of the regular amount of calcium chloride crystals was used. Dale found that irregular contractions of the uterus *in vitro* were less likely to occur when less calcium was used in the solution. We found that when the full amount of calcium was used there was a tendency for the uterus to enter into irregular contractions. The atropine solutions were made up with atropine sulphate in aqueous solutions, having concentration of 1:100. The pituitary extract was used to determine whether the uterine muscle was still irritable to stimulating influences even though it had failed to respond to the antigen following treatment with atropine. For the preparation of crystalline egg albumin we used the methods suggested by Hopkins (10).

## RESULTS

### (*a*) *The specificity of the antigen applied to uteri of virgin guinea-pigs*

In order to determine the degree of specificity and the percentage of sensitization of guinea-pigs in our hands a series of 19 animals were injected with 1 cc. of egg white (1:1), 16 received crystalline egg albumin in salt solution (50 to 200 mgm. each), and another series were treated with 1 cc. of dog serum. Of the 19 pigs injected with egg white the uterine horns of 12 gave a definite response similar to that shown in figure 1 when treated with 0.1 to 0.5 cc. of a 1:10 dilution of egg white in 25 cc. Tyrode's

solution. Fifteen of the 16 receiving crystalline egg albumin were specifically sensitized. In 9 of the 12 cases 0.1 cc. of the egg white dilution produced response. These preparations were all refractory to dog serum or horse serum applied in 0.1 to 0.5 cc. doses of 1:10 dilution. The uteri of the 6 animals sensitized with 1 cc. dog serum all gave definite response to 0.1 to 0.5 cc.

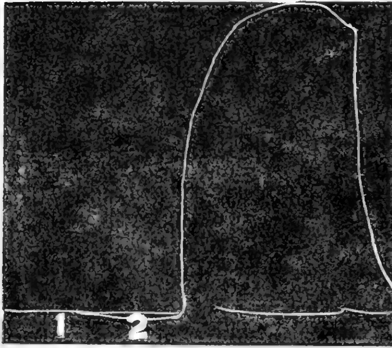


Fig. 1

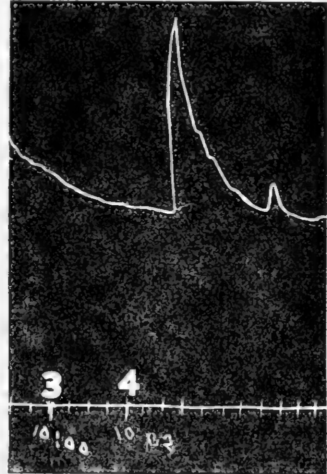


Fig. 2

Fig. 1. Right Uterine Horn of Virgin Guinea-Pig

Sensitized to egg white thirty-five days previous. At *1* added 0.1 cc. dog's serum (1:10) to Tyrode's solution. No response. At *2* added 0.2 cc. egg white 1:10. Showing response.

Fig. 2. Left Horn of Uterus of Virgin Guinea-Pig

Sensitized to dog serum thirty-one days previous. At *3* added 0.5 cc. egg white 1:10. No response. At *4* added 0.1 cc. dog serum 1:10, showing typical response.

of a 1:10 dilution of dog serum and all but one were refractory to a similar dose of egg white or horse serum. Figure 2 shows a tracing which is typical of the series. The uteri were invariably desensitized to the antigen following the first administration. The above results confirm the findings obtained in similar experiments by others.

*(b). The influences of atropine on the reaction of uteri of sensitized guinea-pigs*

The results obtained in this series are tabulated in table 1. Figure 3 shows typical tracings from right and left uterine horns. The animals were injected with egg white, dog serum or horse serum three weeks or longer before the experiment. One horn

TABLE 1

*Effect of atropine on the reaction of one of the uterine horns of sensitized guinea-pigs to an antigen. The opposite horn had in each case shown marked response to a similar or smaller dose of antigen*

| NUMBER | WEIGHT | ANTIGEN | ATROPINE AMOUNT OF 1 PERCENT SOLUTION | ACTION FROM ATROPINE | REACTION FROM ANTIGEN |
|---|---|---|---|---|---|
| | | | *cc.* | | |
| 2 | 370 | Egg white | 1 | Increased tone | No reaction to egg white 2 cc. (1:10) |
| 4 | 350 | Egg white | 1 | Increased tone and contraction | No reaction |
| 9 | 300 | Egg white | 2 | Increased tone | No reaction to 1 cc. egg white (1:10) |
| 10 | 315 | Egg white | 2 | Increased tone | No reaction to 0.14 cc. egg white |
| 14 | 355 | Egg white | 1 | Increased tone and contraction | No reaction to 1 cc. egg white |
| 15 | 450 | Egg white | 1 | Increased tone | No response to 0.15 cc. egg white |
| 17 | 360 | Egg white | 1 | Increased tone | No reaction to 0.2 cc. egg white (1:10) |
| 20 | 325 | Dog's serum | 1 | Increased contractions | No response to 0.1 cc. dog's serum (1:10) |
| 21 | 415 | Dog's serum | 1 | Increased tone | No response to 1 cc. dog's serum (1:10) |
| 24 | 325 | Dog's serum | 1 | Contractions and decreased tone | No response to 1 cc. dog's serum (1:10) |

of each uterus was used for the atropine effect and the other to determine sensitization. Only sensitized animals as shown by response of the uterus to the specific antigen are recorded in this table. The amount of antigen introduced into the Tyrode's solution surrounding the smooth muscle under the influence of atropine was generally applied in larger doses than was necessary

to produce response in control preparation. It will be seen from the table that the atropine in every case prevented the action of the reacting dose of egg white or dog serum. That the atropine did not paralyze the smooth muscle in these cases is shown by the fact that toxic doses of the antigen occasionally produced contractions and that the uteri always responded to a stimulating dose of pituitary extract. Figure 4 gives tracings obtained in such an experiment. Doses of atropine up to 2 cc. of a 1 per cent

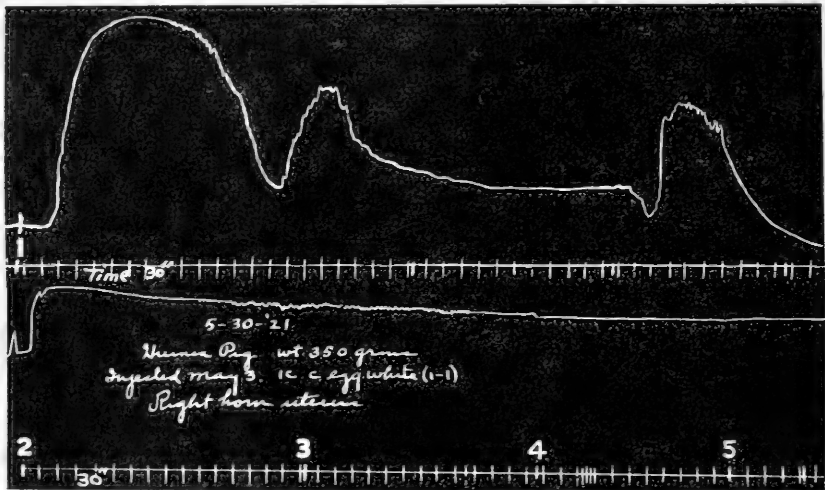


FIG. 3. RIGHT (LOWER) AND LEFT (UPPER) HORN OF UTERUS OF 350 GRAM GUINEA-PIG SENSITIZED TO EGG WHITE

Upper tracing *1* shows response to 0.1 cc. 1:10 egg white. Lower tracing at *2*, 2 cc. atropine 1 per cent; at *3*, 0.1 cc. 1:10 egg white was added. No response. At *4* and *5*, 0.3 cc. and 1 cc. 1:10 egg white was added with no response in either case. Time record thirty seconds.

solution invariably produced an increased tone of the uterine muscles. To eliminate this factor larger doses of atropine, 4 cc. of 1 per cent solution were used. The larger doses generally produced no change in tone although in a few cases there occurred increase or decrease in tone. The smooth muscle was always refractory to reacting doses of the antigen following the larger doses and always reacted to pituitrin.

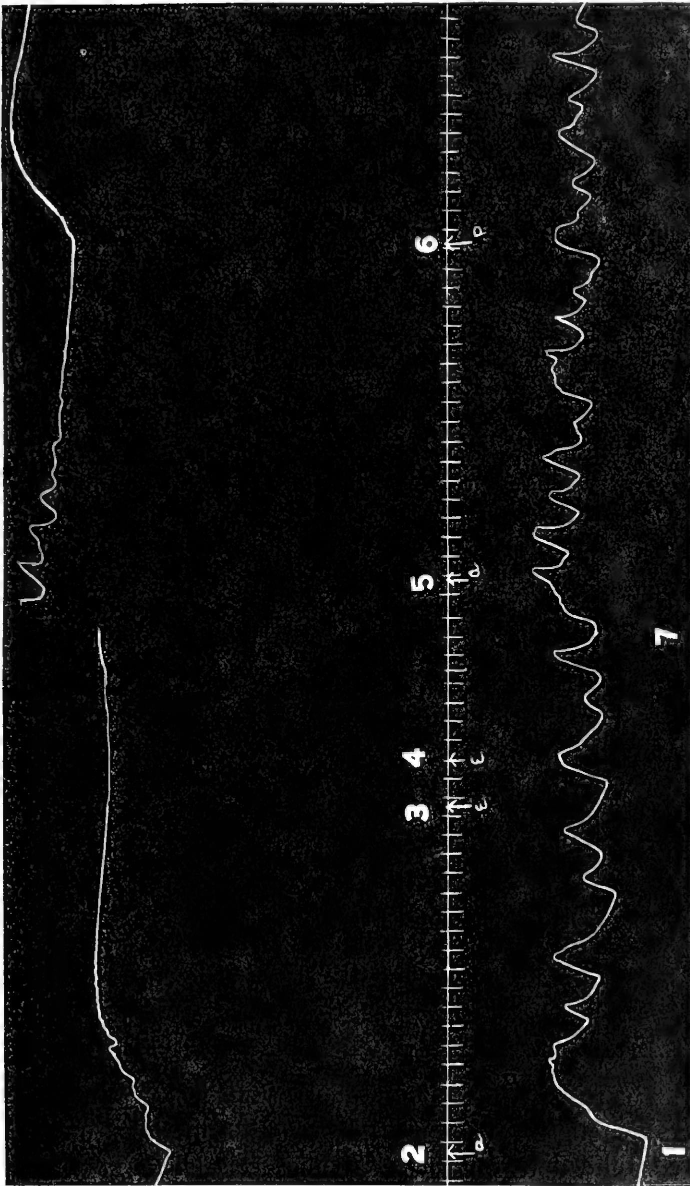


Fig. 4. Left (Upper) and Right (Lower) Horns of Uterus of Guinea-Pig Sensitized with 50 Mgm. Pure Egg Albumin Eighteen Days Previous

Upper tracing, at *2*, 4 cc. atropine 1 per cent added, at *3* and *4*, 0.2 and 1 cc. egg albumin 1:10 was added; no response, showing that atropine desensitized the preparation. Time interval from *4* to *5*, nineteen minutes. At *5*, 4 cc. atropin 1 per cent added. At *6*, 1 cc. pituitrin added. The response to this shows that the atropine does not paralyze the uterine muscle to stimulants other than antigen.

Lower tracing, at *1*, 0.2 egg albumin 1:10 was added. At *7*, 1 cc. of the same dilution. Response at *1* shows that the animal was sensitized to the antigen and failure to respond at *7* twelve minutes later shows desensitization. The preparation later responded to 1 cc. pituitrin.

*(c). The reaction of normal guinea-pig uteri under the influences of atropine to toxic doses of proteins*

The results obtained in this series of experiments are recorded in table 2. Figure 5 shows a typical tracing. The results are not so conclusive as with sensitized preparations. Some response to the protein following the administration of atropine generally occurred although in some cases the action of the protein was negative. The data obtained in the series show that the toxic protein reacts with a different part of the mechanism of the uterus than in the case of the anaphylactic reaction.

TABLE 2

*Effect of atropine upon the reaction of uteri from non-sensitized guinea-pigs to toxic doses of protein*

| GUINEA-PIG NUMBER | WEIGHT | ATROPINE 1 PER CENT ADDED TO 25 CC. TYRODE'S SOLUTION | ACTION OF ATROPINE | REACTION TO ANTIGEN |
|---|---|---|---|---|
| | *grams* | *cc.* | | |
| 27 | 410 | 1 | Rise in tone | No reaction to 0.2 straight dog serum or 0.2 straight egg white |
| 28 | 240 | 1 | Marked increase in contraction and slight rise in tone | Slight response to 0.5 cc. straight dog serum |
| 29 | 240 | 0.25 | Rise in tone and increase in contraction | 3 cc. egg white gives response |
| 30 | 300 | 1 | Marked rise in tone and increase in contractions | Response to 0.2 cc. dog serum |
| 31 | 245 | 0.25 | Rise in tone and increased contractions | Response to 3 cc. egg white |
| 32 | 200 | 0.25 | Rise in tone | 0.3 cc. dog serum gives response |
| 33 | 435 | 0.25 | Give rise in tone | 3 cc. egg white gives response |
| 34 | 324 | 1 | Rise in tone and increase in contractions | Response to 5 cc. dog serum (1:10) |
| 35 | 320 | 1 | Rise in tone | Very slight response to serum |

That the response is due to toxic by-products and not to the protein was demonstrated by some experiments with purified egg white. Large doses of purified egg albumin failed to produce contraction in the uteri of virgin guinea-pigs before or after

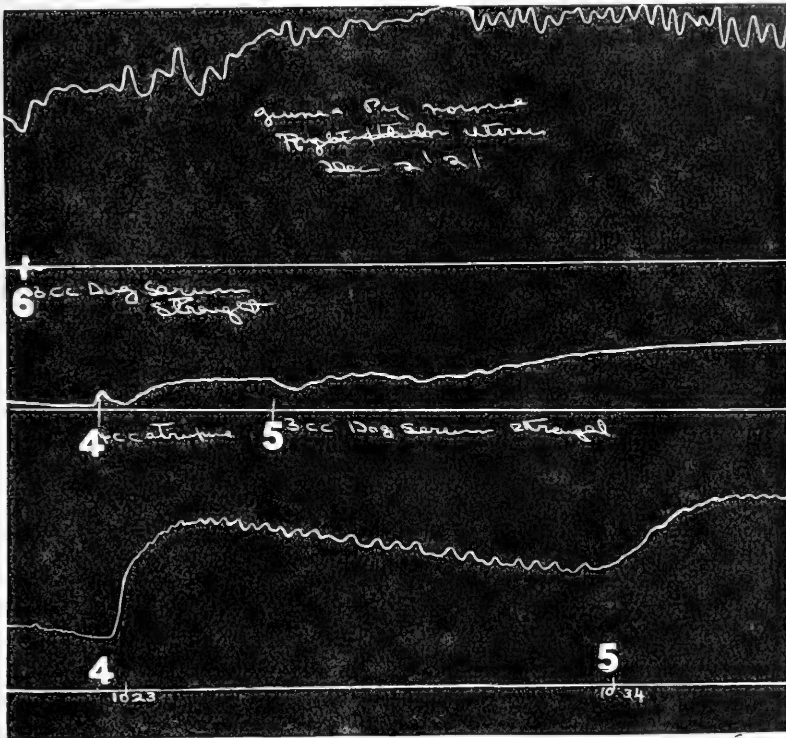


Fig. 5. Uterus of Normal Virgin Guinea-Pig

Upper curve at *6*, 3 cc. dog's serum.
Middle curve at *4*, 4 cc. atropine 1 per cent at *5*, 3 cc. dog's serum.
Lower curve at *4*, 1 cc. atropine 1 per cent at *5*, 5 cc. dog's serum.

atropine administration provided the albumin was free from magnesium or ammonium sulphate used in the purification. We found that when there was a response to purified albumin it could be accounted for by the action of the salts present.

## DISCUSSION OF RESULTS

From the foregoing data, it will be observed that small doses of atropine, i.e., doses ranging from 0.25 cc. to 2 cc. of 1:100 very commonly cause a marked rise in tone, but that the strips were still susceptible to an additional response when pituitary was added. That while in this condition as a result of the effect of atropine, no response was obtained from a reacting dose of the specific protein and very slight or no effect from toxic doses so long as the tone remained at the high level. In normal pigs when there was a drop in tone following the initial rise produced by atropine, there was an additional response to a toxic dose of protein. This did not occur in our experiments except when the tone had dropped below the first level attained after the addition of atropine. When as much as 4 cc. of 1 per cent atropine was added to the same there is very little change in tone either in strips from sensitized or normal guinea-pigs. The muscles, however, are refractory to the toxic response to large doses of native proteins. That these doses of atropine do not paralyze the muscle is indicated by response to the pituitary. One explanation that might be offered for the action of small doses in preventing specific or toxic response is that the atropine stimulated the augmentor mechanism and produces practically the same degree of response that is produced by either small doses of the specific protein or toxic doses of the native proteins, and so long as the tone is at this level, the latter substances would not show any apparent effect.

In other words, the apparent protective effect of small doses may be more apparent than real and perhaps entirely different from the protecting effect of large doses in which there is no increase in tone. This is probably explainable upon the basis of paralysis of the intrinsic mechanism. That the cells are not paralyzed as Auer found in his study of the effect of atropine on the smooth muscle of the lungs, is suggested by the response which the uterine strips gave to stimulation by pituitary extract. Occasionally it was observed that the addition of toxic doses of egg-white to normal strips produced a rise and that when

atropine was added relaxation occurred. In conditions like this, the muscle was susceptible to toxic action of egg-white.

The work of Auer (6) which has been previously cited is perhaps the most notable recent piece of research that has been carried out in an attempt to explain the mechanism of the specific smooth muscle response in the guinea-pig. In analyzing his experiments and results it is interesting to note that the sectioning of either the right or left vagus nerve and occasionally of both vagi either before or after sensitization had no effect upon specific response of the muscle of the lungs. This, however, as Auer is apparently aware, is of value only in that it shows that the effect is either upon the muscle or upon the nervous mechanism between the muscle and the point of sectioning or upon the nervous mechanism outside of the vagi. Auer felt that there is very little experimental evidence to support the latter contention.

In an effort to determine whether the protein acted upon a nervous mechanism or the muscle proper, Auer resorted to nervous degeneration experiments which were evidently thoroughly and satisfactorily performed. That is, he resected the vagus and permitted degeneration to occur for periods ranging from thirty to fifty-seven days and then inoculated with a toxic dose of protein. The animals responded normally. Concerning the interpretation of this work, it would seem that the results which Auer obtained concerning the occurrence of crossing over fibers from the vagus of the opposite side might be of importance. His tracings show that occasionally in a sensitized guinea-pig with both vagi cut the stimulation of the right peripheral vagus produced marked constriction in the left lung, thus showing that each lung probably receives motor fibers from the opposite vagus in a certain per cent of animals. This, along with a careful scrutiny of tracings given, would seem sufficient to cause one to hesitate to draw any sweeping conclusions and would apparently leave the question still an open one as to whether the nervous mechanism played a rôle in the smooth muscle response. It may well be that one of the greatest difficulties involved in the study of this problem is that to date the exact nature of the myoneural junction or receptive substance is not known,—that is, one is apt to speak of muscle and nerve without realizing that

they may not be so sharply differentiated at the point of contact as might be supposed from a casual reading of the literature. In the tracings which we have obtained here our findings would seem to lend themselves to the following interpretation: Relatively large doses of atropine prevented the specific response without apparently affecting the tone. That these muscles were not paralyzed would seem to be indicated by their response to pituitary extract. That is, after the contractile substance of a muscle is paralyzed it would not seem possible to produce further stimulation by pituitary. In order that we can make ourselves clear upon this question we will differentiate between anatomical nerve ending, myoneural junction and contractile substance of the muscle cell. Apparently, then, atropine blocks or paralyzes something that is necessary for the response and that is not the contractile substance of the muscle cell. Ordinarily atropine acts as an antagonist to pilocarpine and from our work it would seem that pilocarpine probably affects that part of the mechanism which is also affected by the specific protein and that this is what is paralyzed by the atropine. In other words, we feel that we have sufficient evidence to warrant the assumption that in addition to any stimulation which specific protein might have upon the muscle cell direct, under favorable conditions, the thing that is directly stimulated in the uterine strips experiments *in vitro* is the receptive substance and probably that part of it known as the myoneural junction. We realize that our experiments concern only excised uterine strips of virgin guinea-pigs and that one is not warranted in making sweeping comparisons between experiments *in vitro* and experiments *in vivo*. Dale and Laidlaw ('11) and others have shown that there is a difference in the effect of drugs upon the uterus *in situ* and the excised uterus. That while pilocarpine inhibits the uterus *in situ*, it stimulates the excised uterus. This has been explained as due to the stimulation of the adrenals by the pilocarpine. We also are aware that the method of using antagonistic action of drugs in locating the point of action of any drug has been the subject of much criticism and must be used with great care. However, in choosing pituitary extract to follow that of atropine, it would appear that this criticism, so far as our knowledge consists at

present, would not invalidate the results or inferences which we have drawn. For that reason we wish our conclusions considered as applying strictly to the excised uterine strips of virgin guinea-pigs. This, of itself, renders it difficult to make use of the data which we have obtained in criticising the work of Auer.

## SUMMARY AND CONCLUSIONS

1. That the response of uteri from sensitized guinea-pigs is specific for antigen has been verified.

2. Doses of atropine from 1 to 4 cc. of a 1 per cent solution added to 25 cc. Tyrode's solution prevents the action of otherwise reactive doses of an antigen when applied to the horns of the uterus of a virgin guinea-pig. Smaller doses of atropine produce an increase in the tone of the uterine muscle, but larger doses (4 cc. 1 per cent) inhibit spontaneous contractions or decrease the tone slightly.

3. That the atropine does not paralyze the uterine muscle is shown by the fact that pituitrin added to the solution invariably results in contraction following the administration of atropine.

4. It is suggested that the antigen acts upon the same mechanism in the neuromuscular complex of the uterus as does pilocarpine. Its action is therefore neutralized by atropine.

5. From the foregoing data we would seem warranted in assuming that that portion of the neuromuscular mechanism known as the myoneural junction plays an important rôle in specific response of uterine strips from sensitized virgin guinea-pigs.

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# FURTHER STUDIES UPON THE COMPLEMENT FIXATION TEST IN CHRONIC GONORRHEA IN WOMEN

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These studies were undertaken in 1919 in coöperation with the Division of Venereal Diseases of the New York State Department of Health with the purpose of determining the value of the complement fixation test, as compared with other laboratory examinations, for the diagnosis of gonorrhea in women. The development of a clinical classification and improvements in method for smear, culture and complement fixation diagnoses were accomplished during the first year. The clinical classification and a comparison of diagnoses by smear, culture and complement fixation in 50 cases of mild, chronic gonorrhea in women were given in our preliminary report (1). Since then the time has been devoted to attempts to improve further the technic of the complement fixation test and to obtain an answer to the following questions: "Is the gonococcus complement fixation test specific?" "Is it an aid to the clinician in diagnosis?"

In attempting to answer the first question it is necessary to take up in detail the various factors in the test and, in discussing the possible defects, to show also the efforts that have been made to overcome some of these defects.

Although the second question is, in part, dealt with by tables 3 and 4, it is answered more fully in clinical papers published by Emily Dunning Barringer and E. von Bose (2) and by Barringer and Williams (3).

## EXPERIMENTS TO IMPROVE THE GONOCOCCUS COMPLEMENT FIXATION TEST

The technic of our diagnostic test was described briefly in our preliminary report (1) and later a detailed account of the method with interpretations of all the reactions was published in Pathogenic Microörganisms by Park and Williams (4). This method had been adopted by us as a standard before the present study was begun and has been continued as a standard, or control, in our attempts to improve the test.

Our experiments include:

Investigation of various methods for the preparation of antigen.

Tests as to the anticomplementary reaction of the patient's serum and the presence of natural antisheep amboceptor.

Standardization of complement.

### ANTIGEN

Many experiments have been undertaken by us to develop an antigen that will give a higher percentage of positive complement fixation reactions with serums of gonorrheal patients. These experiments dealt with the antigenicity of the gonococcus, the advantage of polyvalent antigens and the comparison of various culture media for antigen production.

#### *Comparison of the antigenicity of the Torrey strains with recently isolated gonococci*

In 1916 Dr. Archibald McNeil invited our coöperation in his study of antigen preparations. We isolated gonococci from 28 cases of acute urethritis in the male and made antigens of the new cultures at the same time and upon the same medium as the antigens prepared with the Torrey strains (5). Comparative tests of all the antigens were made with gonococcus immune rabbit serums and human gonorrheal serums. The freshly isolated cultures proved to be of lower antigenicity than the Torrey strains when tested with the same serums. The antigens from freshly isolated cultures had to be used in 1:10 dilution to

obtain the same reactions that occurred with the Torrey strains in 1:20 dilution. The new isolations have less growth in the forty-eight hours than the Torrey strains, but the suspensions of the new cultures before being diluted and the suspensions of the Torrey strains before dilution were macroscopically of the same density, and all the antigen suspensions were made by weighing the dried cocci and adding saline in the proportion of 1 gram of dried cocci to 200 cc. of saline solution.

At the beginning of our present study, Miss Kutner isolated gonococci from 10 cases of gonorrhea and prepared an antigen from the cultures. These cases included both male and female adults.

Our complement fixation tests with her antigen were similar to those obtained with our antigens made from freshly isolated cultures.

In 1920 we isolated gonococci from 42 cases of gonorrheal vaginitis and made several polyvalent antigens. These, also, gave the same reactions in 1:10 dilution that the Torrey strain antigens gave in 1:20 dilution, thus, confirming the results of our two previous studies.

These experiments convinced us that the Torrey strains had not lost their antigenicity, and we have continued to use them for our stock antigens.

*Will autogenous antigens and other monovalent antigens detect gonococcus antibodies that are missed by the Torrey strains?*

Up to the present time we have given very little study to this subject, but are planning to follow it more fully in the future.

We have made three monovalent antigens: one from a culture isolated from acute gonorrheal vaginitis (Pecchio), one from a gonococcus isolated from Bartholin's gland (Dolson), and one from the blood of a scarlet fever patient having the complication of gonorrhea (Fazzio). The Dolson and Fazzio cultures were identified as gonococci by positive agglutination reactions in high dilution.

The agglutination tests were made by Dr. John C. Torrey.

The Pecchio antigen did not give a complement fixation reaction with the Pecchio human serum, but gave complete fixation, in 1:10 dilution, with a polyvalent serum from a rabbit immunized with the ten Torrey strains. The Pecchio human serum gave a weakly positive reaction with the Torrey polyvalent antigen. The Dolson monovalent antigen gave the same weakly positive reaction with its homologous serum as that serum gave with the Torrey polyvalent antigen and with the Fazzio monovalent antigen. These tests were made at the same time and with the same reagents. There was insufficient antigen prepared to continue the studies and the cultures were lost before another lot of antigen could be prepared. The following table shows these reactions:

TABLE 1

*Complement fixation with monovalent and polyvalent antigens*

| SERUMS | ANTIGENS | | | |
|---|---|---|---|---|
| | Monovalent | | | Polyvalent |
| | Pecchio dilution 1:10 | Dolson dilution 1:10 | Fazzio dilution 1:10 | Torrey strains dilution 1:20 |
| Pecchio (human) | Negative | No test | No test | Weakly positive |
| Dolson (human) | No test | Weakly positive | Weakly positive | Weakly positive |
| Fazzio (human) | No test | Strongly positive | Strongly positive | Strongly positive |
| Control. Rabbit immunized with Torrey's ten gonococcus strains | Strongly positive | Strongly positive | Strongly positive | Strongly positive |

In the above tests it will be seen that the Pecchio serum did not react with its own antigen, but did react with the Torrey antigen.

The Dolson and Fazzio serums gave the same reactions with their own antigens that they did with the Torrey antigen. The rabbit serum (Torrey strains) reacted with all the antigens.

*Effects of culture media upon antigenicity of the gonococcus*

We have made polyvalent antigens with the ten Torrey strains upon various media, including salt-free-veal-agar, Vedder's starch medium, glucose-ascitic-veal-agar, vitamin-agar, glycerine-horse-serum-veal-agar, and disodium-phosphate-agar. Our findings are that the antigenicity of the gonococcus is not affected by the culture medium if the medium gives an abundant growth within forty-eight hours. Antigens made from twenty-four-hour, or seventy-two-hour growths, have to be used in lower dilution than those made from forty-eight-hour growths. However, although the antigenicity of the gonococcus is not affected by the culture medium, its anticomplementary property may be increased. We have found the antigens made from growth on Vedder's starch medium to be anticomplementary. Iodin tests revealed starch granules after the cocci had been washed many times to free them from the starch.[1] Media containing hemoglobin, also tend to produce an anticomplementary antigen. We have had uniformly good results with antigens made from growth on glycerine-horse-serum-veal-agar and on disodium-phosphate-agar. A small volume of antigen from growth on wheat medium was tried. There was not enough of the wheat medium antigen to test the human serums, but this antigen gave the same reaction with the gonococcus immune-rabbit-serum that the disodium-phosphate-agar antigen did.

We are planning a more complete study of different methods of antigen production.

THE PATIENT'S SERUM

Two factors tend to give "false" reactions with the patient's serum: the anticomplementary reaction of the serum and the presence of natural antisheep amboceptor. The former gives a so-called false positive reaction, and the latter a false negative reaction.

*Anticomplementary serums*

In this laboratory we have had less than 1 per cent of anticomplementary patients' serums that could not be controlled by heat-

[1] The tests for starch were made by Dr. McNeil.

ing. The most notable example was a serum from a sixteen-year-old girl whose clinical diagnosis was "acute gonorrhea." This patient was bled at intervals of one week for thirteen weeks. The bleedings were tested in two laboratories and all reports were returned as "anticomplementary."

We tried a number of experiments to overcome this anticomplementary property. Upon the theory that the anticomplementary reaction in the patient's serum is due to the presence of both antigen and antibody in the right proportion to absorb complement, we tried to break down the antigen-antibody combination by adding either an excess of antigen, or an excess of antibody, but none of our experiments eliminated the anticomplementary property.

Active and inactivated serums gave the same anticomplementary reaction. The simple method recommended by Breuer (6) was not tried on this serum, as his article had not been published at that time and his procedure had not been thought of by us. Breuer used a constant volume of patient's serum with varying amounts of complement and found that he could always obtain some dose of complement that would enable him to make a diagnosis of serums that would be anticomplementary with the usual dose of complement.

*The presence of natural antisheep amboceptor in the patient's serum*

The question of natural antisheep amboceptor is one to be considered. In table 2 are tabulated the results in a study of 2374 bleedings from 753 patients. The incidence of natural antisheep amboceptor that gave a negative reaction in a positive serum was only 0.3 per cent. In 60.6 per cent of these 2374 bleedings there was enough natural antisheep amboceptor to to hemolyze from one-fourth to three-fourths of the sheep cells in the hemolytic system. In 50 per cent of the bleedings represented by the 60.6 per cent the small content of natural antisheep amboceptor did not interfere, apparently, with the complement fixation reaction. This conclusion was drawn from the fact that a human bleeding of one week might have no natural antisheep

amboceptor and would give a weakly-positive reaction, while on the following week a bleeding from the same patient might have enough natural antisheep amboceptor to hemolyze half the cells and continue the weakly-positive reaction.

On the other hand, a number of serums having enough natural amboceptor completely to hemolyze all the cells in the hemolytic system have given a strongly positive reaction. In a recent example of this occurrence, the diagnostic test continued to be four-plus after standing in the water-bath for one hour, and then overnight in the ice-box.

As a control for natural antisheep amboceptor, we have used the Kaliski (7) (8) modification of the Bauer (9) test, in which the natural antisheep amboceptor in the patient's serum is utilized for completing the hemolytic system, and, in the event of there being no antisheep amboceptor, or less than one unit, enough artificial amboceptor is added to produce complete hemolysis in the hemolytic system.

We have found the Kaliski method to be inadequate as a diagnostic test for the reason that a completely hemolyzed system is seldom obtained when artificial amboceptor is added to supplement the natural antisheep amboceptor.

The nature of the natural antisheep amboceptor reaction has not been explained sufficiently to enable one to control it absolutely. That an antigen in association with its homologous antibody can be dissociated from that antibody by the addition of an excess of artificial antisheep amboceptor is illustrated by the following example:

*Dissociation of antigen from its homologous antibody*

First period of the test

| REAGENTS | DIAGNOSTIC TEST | | | | KALISKI-BAUER CONTROL | | | |
|---|---|---|---|---|---|---|---|---|
| | Serum control | | Serum + antigen | | Serum control | | Serum + antigen | |
| Serum | 0.04 | 0.02 | 0.02 | 0.01 | 0.04 | 0.02 | 0.02 | 0.01 |
| Antigen | None | None | 0.1 | 0.1 | None | None | 0.1 | 0.1 |
| Complement | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Fixation period: one hour in water-bath at 37°C.

The foregoing chart shows the contents of all the tubes in the complement fixation tests up to the end of the fixation period. It will be noticed that the diagnostic test and the Kaliski-Bauer control contain exactly the same reagents in the same amounts. They are both fixed at the same temperature for the same time. Then, to the diagnostic test is added the standard dose of sensitized cells. (This dose contains 0.1 cc. of a 5 per cent suspension of sheep cells and two hemolytic units of antisheep amboceptor.) To the Kaliksi-Bauer control is added 0.1 cc. of a 5 per cent suspension of sheep cells without any artificial amboceptor. After the cells have been added to all the tubes, the tests are replaced in the water-bath for the second incubation and at the end of ten minutes the reactions are read. The following chart shows the reactions in the tubes at the end of the second incubation period, after the addition of sensitized cells to the diagnostic test and 5 per cent suspension to the Kaliski-Bauer control:

Second period of the test After ten minutes in water-bath

| | DIAGNOSTIC TEST | | KALISKI-BAUER CONTROL | |
|---|---|---|---|---|
| | Serum control | Serum + antigen | Serum control | Serum + antigen |
| Reaction | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed | Completely fixed |
| Diagnosis | Negative | | Four-plus Interpreted as strongly positive | |

As the above test and control were identical up to the end of the fixation period, if the complement were fixed as shown in the reading of the Kaliski-Bauer control, it must, also, have been fixed in the diagnostic test. Therefore, we conclude that the presence of natural antisheep amboceptor in the patient's serum did not interfere with the fixation of complement, but, that the addition of an excess of antisheep amboceptor dissociated the gonococcus fixation amboceptor in the patient's serum from the gonococcus antigen, thus, liberating the complement that had been completely fixed and giving a negative reaction. This point continues to be a subject for study in our laboratory.

The absorption of natural antisheep amboceptor has been recommended by some writers (10) (11) (12) (13), but we have not found it to be an adequate remedy.

Table 2 shows the incidence of natural antisheep amboceptor in 2374 bleedings.

TABLE 2

*Showing the incidence of natural antisheep amboceptor in patients' serums*

<table>
<tr><th rowspan="4">NUMBER OF BLEEDINGS</th><th colspan="3">PERCENTAGE HAVING ONE UNIT, OR MORE, OF NATURAL ANTISHEEP AMBOCEPTOR</th><th rowspan="4">PERCENTAGE HAVING LESS THAN ONE UNIT OF NATURAL ANTISHEEP-AMBOCEPTOR</th><th rowspan="4">PERCENTAGE HAVING NO NATURAL ANTISHEEP-AMBOCEPTOR</th></tr>
<tr><th colspan="3">1.3</th></tr>
<tr><th colspan="3">These are subdivided into:</th></tr>
<tr><th>Per cent positive reactions reduced to negative</th><th>Per cent positive reactions not changed</th><th>Per cent doubtful reactions</th></tr>
<tr><td>2374</td><td>0.3</td><td>0.1</td><td>0.9</td><td>60.6</td><td>38.1</td></tr>
</table>

## COMPLEMENT

The source of greatest difficulty in the performance of the complement fixation test lies in the variation of the guinea-pig complement. As we have shown in our preliminary report (1), the guinea-pig serums vary in fixability as well as in hemolytic activity, and before being pooled for tests all guinea-pig serums must be tested for natural antisheep amboceptor, hemolytic activity, anticomplementary reaction with the gonococcus antigen, anticomplementary reaction with the control gonococcus serum, and for fixability with the gonococcus antigen-serum-complex. The details of these preliminary tests and their interpretation have been published (4).

During the year 1920, out of 481 guinea-pigs tested, only 57 per cent could be used for gonococcus complement fixation.

### *Hemolytic system*

After selecting the complement by the preliminary tests, the pooled complement is titrated with sensitized cells in order to determine the exact dose of pooled complement to be used for tests.

The sensitized cell dose contains two hemolytic units of anti-sheep amboceptor and 0.1 cc. of 5 per cent suspension of sheep cells.

The complement unit is read at the end of thirty minutes in the water-bath at 37°C. We use two hemolytic units of complement for diagnostic tests.

*The diagnostic test*

We use one-tenth the original Wassermann (14) volumes of all reagents.

The diagnostic tests are made in duplicate, with controls for the hemolytic system, for anticomplementary reaction of the patient's serum, for natural antisheep amboceptor in the patient's serum, and for fixation unit and anticomplementary reaction of the gonococcus antigen.

The diagnostic test and all controls are fixed in the water-bath for one hour at 37°C.

*Reading.* The following controls must be completely hemolyzed before the diagnostic tests are read: system control, control for anticomplementary reaction in patient's serum, the antigen anticomplementary control (0.4 cc. of the antigen dilution must be completely hemolyzed, that is, four times the dose used in the test).

The average complement used throughout this study has given complete hemolysis in all controls in twelve minutes.

*Diagnosis.* If the patient's serum contains no natural anti-sheep amboceptor and if it is not anticomplementary, the diagnosis is made in accordance with the reactions in the tubes containing antigen plus serum. The Citron (14) method for readings is used, with the exception of our "strong-one-plus," "one-plus" and "plus-minus" reactions, which Citron does not mention in his description of reactions.

Our "strong-one-plus" has very strong fixation in both the 0.02 and 0.01 tubes of the test; our "one-plus" has strong fixation in the 0.02 and weak fixation in the 0.01; our "plus-minus" has partial fixation in both the 0.02 and 0.01 tubes.

*Anticomplementary serums.* If the control for anticomplementary reaction in the patient's serum is not completely hemolyzed at the time required for hemolysis of the other hemolytic controls, the test is replaced in the water-bath until the end of one hour. If at that time the serum still shows any degree of anticomplementary reaction, no diagnosis is made of that bleeding, and it is reported as "anticomplementary."

*Natural antisheep-amboceptor.* If the diagnostic test is negative and the Kaliski-Bauer control shows a typical four-plus reaction, we report the test as: "Four plus by Kaliski-Bauer control." If the diagnostic test is negative and the Kaliski-Bauer control shows less than a four-plus reaction, we report the test as: "Negative. Serum contains an excess of natural antisheep amboceptor." In such cases we request another bleeding. If the serum controls in the Kaliski-Bauer control have partial hemolysis, we report the test according to the diagnostic test, regardless of the small amount of natural antisheep amboceptor. In table 2 we have shown that the presence of natural antisheep amboceptor reduced a positive reaction to negative in only 0.3 per cent of 2374 bleedings. Although this seems an insignificant number, we continue to use the control for the sake of the individual, and to give us an explanation of variation in the complement fixation curves of patients bled week after week.

*Interpretation of diagnosis.* All tests showing complete fixation in the 0.01 tube are interpreted as "very strongly positive." This is the Citron "four-plus" reaction, and is his interpretation.

All tests showing strong fixation in the 0.01 tube are interpreted as strongly positive. This includes Citron's "three-plus" and our "strong one-plus" reactions, and is Citron's interpretation of his "three-plus." In our "strong one-plus" reaction the 0.02 and 0.01 tubes are both very strongly fixed. Citron does not mention this reaction, but it is not infrequently encountered in the tests in this laboratory and must be considered in an accurate interpretation of diagnoses. It is stronger than Citron's "two-plus" and a shade less (in the 0.02 tube only) than Citron's "three-plus."[2]

[2] Unpublished report of the Research Laboratory Committee on Standardization of the Wassermann Test.

All tests showing strong fixation in the 0.02 tube and weaker fixation in the 0.01 tube are interpreted as moderately positive. This is our "one-plus" reaction. It is stronger in the 0.01 tube than Citron's "two-plus," and a shade less in the 0.02 tube than his "two-plus." In our interpretation of moderately positive reactions, we include the Citron "two-plus." The latter is infrequently encountered in our tests, while our "one-plus is of frequent occurrence. Citron does not describe our "one-plus."

All tests in which the 0.02 and the 0.01 tubes show partial fixation, (from 25 to 50 per cent hemolysis), are interpreted as weakly positive. This is our "plus-minus" reaction. It is stronger in the 0.01 tube than Citron's "two-plus" and has the same degree of fixation in the 0.02 tube as his "one-plus." This reaction is not described by Citron, but is of frequent occurrence in our tests.

In our weakly-positive interpretation we include Citron's "one-plus."

All tests showing 75 per cent or more hemolysis in the 0.02 tube are interpreted as "doubtful."

Our interpretation is the result of the study of a large number of tests in relation to the clinical classification.

In cases clinically diagnosed as negative for gonococcus we have obtained no fixation, not even a doubtful reaction, and for that reason we feel justified in placing in the positive column the reaction described above.

## SPECIFICITY OF THE GONOCOCCUS COMPLEMENT FIXATION TEST

In this study we have examined repeated bleedings from 870 gonorrheal cases controlled by bleedings from 350 non-gonorrheal individuals. In testing the specificity of the gonococcus antibody 92 of the gonorrheal bleedings were tested with tuberculosis antigen and gave negative reactions, and 54 of the gonorrheal bleedings were tested with *Streptococcus viridans* antigen and gave negative reactions. We have not included the Wassermann antigen in our controls of specificity of antibody for the reason that a considerable number of the patients in this service have a clinical diagnosis of syphilis, or doubtful for syphilis, as well as of gonorrhea.

TABLE 3

*Gonococcus complement fixation in various stages of gonorrhoea in women*

| CLINICAL DIAGNOSIS | NUMBER OF CASES | GONOCOCCUS COMPLEMENT FIXATION | | | |
|---|---|---|---|---|---|
| | | Positive | Negative | Doubtful | Percentage positive |
| Acute gonorrhea.* | 19 | 9 | 6 | 4 | 47 |
| Subacute gonorrhea | 112 | 54 | 36 | 22 | 48 |
| Chronic gonorrhea | 383 | 283 | 56 | 44 | 73.6 |
| Syphilis and gonorrhea | 162 | 132 | 14 | 16 | 81.4 |
| Syphilis, doubtful for gonorrhea | 113 | 24 | 88 | 1 | 21 |
| Doubtful gonorrhea | 81 | 49 | 21 | 11 | 60 |
| **Controls** | | | | | |
| Adults having no symptoms or history of gonorrhea | 50 | 0 | 50 | 0 | 0 |
| Children: Scarlet fever patients having no symptoms or history of gonorrhea. | 36 | 0 | 36 | 0 | 0 |
| Spinal fluids from poliomyelitis cases | 12 | 0 | 12 | 0 | 0 |
| Adults: Tubercular patients having no gonorrheal symptoms | 248 | 0 | 248 | 0 | 0 |
| Normal bleedings from workers in this laboratory | 4 | 0 | 4 | 0 | 0 |
| Total cases | 1220 | | | | |

* Not *early* acute.

TABLE 4

*Comparison of diagnoses by smear, culture and complement fixation*

| CLINICAL DIAGNOSIS | NUMBER OF CASES | GONOCOCCUS COMPLEMENT FIXATION | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Smear positive, culture positive | | | Smear doubtful, culture positive | | | Smear doubtful, culture negative | | | Smear negative, culture negative | | |
| | | Positive | Negative | Doubtful | Positive | Negative | Doubtful | Positive | Negative | Doubtful | Positive | Negative | Doubtful |
| Acute | 11 | 3 | 2 | 1 | 1 | | | 1 | 2 | | | | 1 |
| Subacute | 43 | 10 | 1 | 3 | 6 | 1 | | 9 | 4 | 3 | | 4 | 2 |
| Chronic | 85 | 9 | 1 | 1 | 2 | | | 39 | 2 | 8 | 15 | 2 | 6 |
| Chronic and syphilis | 35 | 3 | | 1 | | | | 16 | 2 | 5 | 7 | 1 | |
| Doubtful | 7 | | | | | | | 2 | 3 | | 1 | 1 | |
| Total cases | 181 | | | | | | | | | | | | |

In testing the specificity of the gonococcus antigen, bleedings from 350 patients clinically diagnosed as non-gonorrheal gave no trace of gonococcus complement fixation reaction.

Table 3 shows the incidence of gonococcus complement fixation at various stages of the disease and also the specificity tests upon non-gonorrheal serums.

Table 4 shows the comparison of smear, culture and complement fixation in 181 cases. These three laboratory tests are more fully discussed in a paper by Torrey and Wilson (15).

In table 5 is given a summary of the diagnoses reported in table 4, showing how the precentage of positive diagnoses by smear and culture decrease with the stage of the disease and how the percentage of positive complement fixation increases.

TABLE 5

*Summary of diagnoses*

| CLINICAL DIAGNOSIS | NUMBER OF CASES | PERCENTAGE OF POSITIVE DIAGNOSES | | |
|---|---|---|---|---|
| | | Smear | Culture | Complement fixation |
| Acute gonorrhea* | 11 | 55 | 55 | 45 |
| Subacute gonorrhea | 45 | 32 | 44 | 58 |
| Chronic gonorrhea | 85 | 13 | 15 | 76 |
| Gonorrhea and syphilis | 35 | 11 | 11 | 76 |
| Doubtful gonorrhea | 7 | None | None | 42 |
| Total cases | 181 | | | |

* Not *early* acute.

## SUMMARY

Investigation of various media for the preparation of gonococcus antigen showed that any of the starch-free and hemoglobin-free media that would give a profuse growth of the gonococcus within forty-eight hours at 37°C. would give antigens of equal value.

Recently isolated gonococci were of lower antigenicity than the Torrey strains isolated many years ago.

Autogenous antigens gave no stronger reactions with their homologous serums than did the Torrey strain antigen.

In this study we have encountered very few anticomplementary patients' serums that could not be controlled by heating.

The incidence of natural antisheep amboceptor that gave a negative reaction in a positive serum was only 0.3 per cent of 2374 bleedings. We found the Kaliski-Bauer test to be inadequate as a diagnostic measure, but of value as a control. Absorption of natural antisheep amboceptor did not give us uniform reactions.

As previously reported, we found the difference in fixative and hemolytic value of guinea-pig complements to be the greatest factor of variation in the gonococcus complement fixation test.

In order to give a true interpretation of our diagnostic tests in relation to the clinical classification, we have had to include three reactions frequently encountered in our tests that were not mentioned by Citron. Otherwise, our interpretations agree with Citron's.

Specificity tests with heterologous antigens gave no fixation with gonorrheal serums, as shown in table 3.

Non-gonorrheal serums tested with gonococcus antigens gave no complement fixation.

Tables 4 and 5 show the relative value of smear, culture and complement fixation. These three laboratory methods are discussed in a paper by Torrey and Wilson (15).

The clinical value of our gonococcus complement fixation tests is discussed in the publications of Barringer and Williams (3), and Barringer and von Bose (2).

## CONCLUSIONS

1. Individual guinea-pig-serums must be tested for fixability with known gonococcus antibody plus gonococcus antigen before they can be used as complement for tests of patients' serums.
2. The negative results with control human serums throughout this series of tests indicate that the gonococcus complement fixation test is specific.
3. The gonococcus complement fixation test is an aid to the clinician in institutional work (2).

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## II. THE HEPATIC MECHANICAL FACTOR IN PEPTONE SHOCK

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Most of the current theories of canine anaphylactic and peptone shock lay emphasis on the mechanical factor in the liver (1). An increased resistance to blood flow through this organ is the assumed underlying cause of the characteristic clinical picture. The theories differ only in their hypotheses as to the mechanism of the assumed increase in resistance to hepatic blood flow. We therefore have sought to determine whether or not an increased resistance to blood flow can be demonstrated in the liver of dogs during peptone shock, and if so, whether or not this increase is sufficient to account for the characteristic splanchnic engorgement and the characteristic fall in systemic blood pressure.

To do this we have studied: (1) the changes in the portal blood pressure during peptone shock; (2) the changes in the perfusion resistance of isolated livers, produced by peptone; and (3) the local and general circulatory disturbances secondary to a mechanically increased hepatic resistance.

### PORTAL BLOOD PRESSURE DURING PEPTONE SHOCK

To follow the changes in the portal blood pressure, a cannula was tied in the pancreatic vein. This vein connects with the portal vein without valves. Parallel kymograph records were then made of the changes in the carotid and portal blood pressures following intravenous peptone injections. Typical records thus obtained are shown in figure 1.

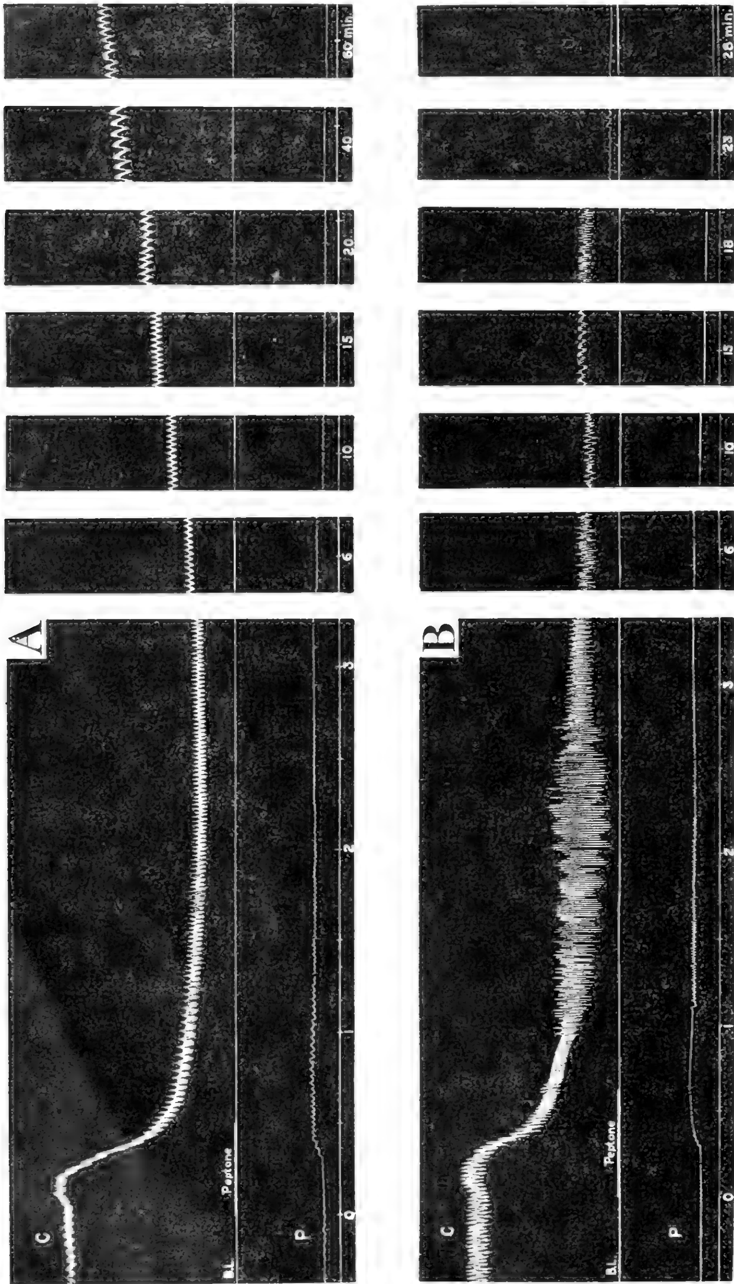


Fig. 1. Changes in Portal Blood Pressure During Peptone Shock

These records show the maximum rise in portal blood pressure observed in our series. $A$ = Typical peptone shock. $B$ = Fatal peptone shock. $C$ = Carotid blood pressure. $P$ = Portal blood pressure.

The normal portal blood pressure in dogs varies from 7 mm. Hg to 13 mm. Hg, with an average of about 9 mm. Hg in our series. Following an intravenous peptone injection, there is a rapid, pronounced fall in carotid blood pressure, the fall beginning within fifteen seconds after commencing the peptone injection, and reaching a level of about 40 mm. Hg by the end of one minute. This fall always preceeds recordable changes in portal blood pressure.

About twenty seconds after beginning the peptone injection, a recordable rise in portal blood pressure is observed. The portal blood pressure usually reaches a maximum by the end of forty-five seconds. This maximum varies from 13 mm. Hg to 22 mm. Hg, depending upon the initial portal pressure, showing a rise of from 6 mm. Hg to 9 mm. Hg, an average rise of 7 mm. Hg in our series.

The portal blood pressure remains at approximately this maximum for about three minutes. It then gradually falls, reaching normal in from eight to twelve minutes. The carotid pressure, meanwhile, decreases slowly to a minimum of about 30 mm. Hg, the minimum usually being reached between the second and tenth minute. After reaching this minimum, the carotid pressure usually rises slowly, reaching normal in from thirty to ninety minutes, depending upon the peptone dose injected. Occasionally, as in tracing *B*, the carotid pressure shows little or no tendency to rise, remaining at about 30 mm. Hg till the death of the animal, death usually taking place in from thirty to forty minutes.

There is therefore a fairly constant relationship between the portal and carotid blood pressures during the first ten minutes of the peptone reaction. The portal blood pressure during this period always increases, but never increases more than 9 mm. Hg above the initial portal pressure. The portal blood pressure never becomes equal to the simultaneous aortic pressure, its maximum usually being about 50 per cent of the aortic pressure. The rise in portal pressure therefore does not necessarily prove an increased hepatic resistance. A rise of from 6 mm. Hg to 9 mm. Hg might conceivably be produced solely by intestinal vasodilation.

PERFUSION RESISTANCE OF ISOLATED LIVERS

Perfusion tests were made with isolated livers and with isolated hepatic lobes using: (1) peptone solutions; (2) peptone-defibrinated-blood mixtures; and (3) peptone-uncoagulated-blood mixtures.

*1. Peptone perfusions.* Quantitative perfusions of the whole liver with Ringer's solution, followed by Ringer's solution containing varying amounts of peptone; technic described in a

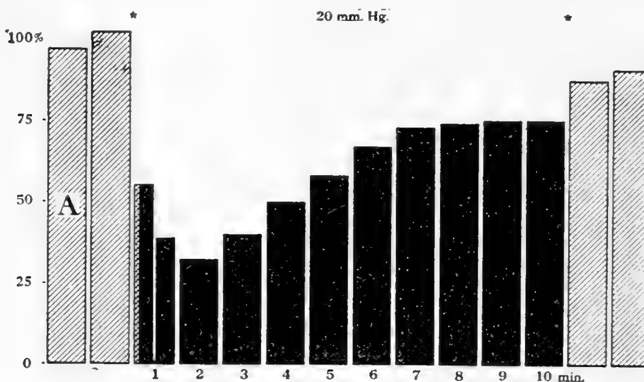


FIG. 2. PEPTONE PERFUSION OF ISOLATED LIVER

Composite data from two perfusions with 0.9 per cent and 1.3 per cent peptone, corresponding roughly to intravenous injections of 0.35 gram and 0.5 gram peptone per kilogram of body weight. Cross-hatched areas represent perfusion flow per minute with Ringer's solution. This rate varies from 800 cc. to 1500 cc. depending upon the size of the animal. Black areas represent perfusion flow per minute (or half-minute) with Ringer's solution containing unneutralized Witte's peptone. Stars (*) show time of changing perfusion clamps. Perfusion pressure, 20 mm. Hg throughout test.

previous paper (1). Typical data thus obtained are shown in figure 2.

Marked quantitative differences were noted in the reactions of the isolated liver to different peptones. With peptone "Difco," for example, and with accurately neutralized Witte's peptone, but slight hepatic reactions were obtained. With unneutralized (alkaline) Witte's peptone, however, marked reactions were produced. In the typical reaction to unneutralized

Witte's peptone, the rate of perfusion flow decreases rapidly during the first half minutes, reaching a minimum by the end of the first minute. The perfusion rate during the second minute varies from 25 per cent to 75 per cent of the normal perfusion rate, depending upon the peptone concentration tested. The perfusion rate increases after the second minute, usually reaching normal by the eighth minute. With large peptone doses (fig. 2) this recovery is only partial.

*2. Peptone-defibrinated-blood perfusions.* Quantitative perfusions of isolated hepatic lobes with defibrinated blood, followed by peptone-defibrinated-blood mixtures. To make these perfusions the hepatic artery was ligated and an unclosed ligature was placed about the vena cava immediately above the right middle hepatic lobe. An afferent cannula was tied in the portal vein, and an efferent cannula in the vena cava immediately below the liver. On tightening the vena caval ligature, the two lower lobes of the liver could thus be isolated and perfused. Typical data thus obtained are shown in figure 3.

In these perfusions no marked differences were noted between the hepatic reactions to different peptones. In all cases the perfusion rate was greatly reduced by the peptone-defibrinated-blood mixture. The perfusion rate usually reached a minimum of about 25 per cent of the initial rate by the end of the second minute, and then gradually increased. The defibrinated blood reactions were always more pronounced than the reactions previously obtained with unneutralized Witte's peptone. On increasing the perfusion pressure to 20 mm. Hg, the rate of perfusion flow was more than doubled.

*3. Peptone-uncoagulated-blood perfusions.* Quantitative perfusions of isolated hepatic lobes with uncoagulated blood, followed by peptone-uncoagulated-blood mixtures. Technique the same as with the defibrinated blood tests, except that in these tests the blood of the donor was allowed to flow continuously into the pressure flask. Typical data thus obtained are shown in figure 4.

In the first test here recorded (*C*) the average perfusion rate of 43 cc. per half-minute was obtained with a preliminary perfusion pressure of 10 mm. Hg. This rate was increased to 88 cc.

per half-minute on increasing the perfusion pressure to 20 mm. Hg. On now adding 1 per cent peptone to the uncoagulated blood the perfusion rate was reduced to 37 cc. per half-minute.

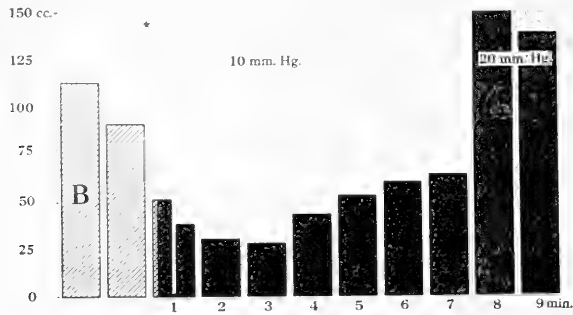


FIG. 3. PEPTONE-DEFIBRINATED-BLOOD PERFUSION OF ISOLATED HEPATIC LOBES

Cross-hatched areas show perfusion flow per minute with defibrinated blood. Black areas show rate per minute (or half-minute) with defibrinated blood plus 1 per cent peptone. Initial perfusion pressure, 10 mm. Hg, increased to 20 mm. Hg at the beginning of the eighth minute.

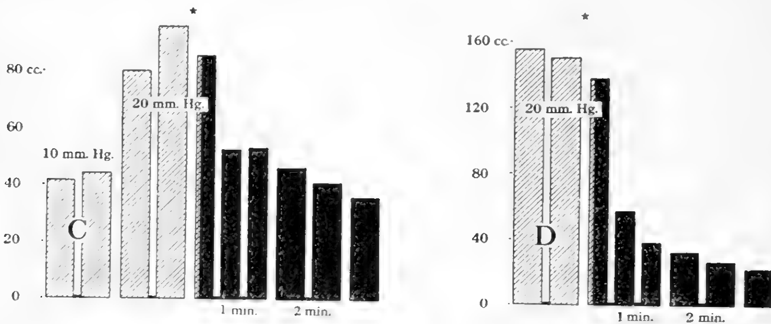


FIG. 4. PEPTONE-UNCOAGULATED-BLOOD PERFUSIONS OF ISOLATED HEPATIC LOBES

Cross-hatched areas show perfusion flow per half minute with uncoagulated blood. Black areas show rate per half minute (or third minute) with uncoagulated blood plus 1 per cent peptone. *C:* Initial perfusion pressure, 10 mm. Hg, increased to 20 mm. Hg before adding peptone. *D:* Perfusion pressure, 20 mm. Hg throughout test.

In the second recorded test (*D*) the average perfusion rate was decreased from 153 cc. to 32 cc. by the peptone addition. In all of our tests the peptone-blood mixtures were rendered non-coagulable by their passage through the liver.

*4. Conclusions.* Our conclusion from these tests is that the resistance to hepatic blood flow is markedly increased during the initial stages of peptone shock. The increase, however, is not sufficient to materially decrease the rate of hepatic blood flow. The increased portal blood pressure fully compensates for the increased hepatic resistance.

## MECHANICALLY INCREASED HEPATIC RESISTANCE

A study was made of the local and general circulatory effects of carefully controlled mechanical obstruction: (1) to the portal vein, and (2) to hepatic outflow.

*1. Mechanically increased portal resistance.* To make these tests, the dogs were prepared for parallel tracings of carotid and portal blood pressure. An unclosed ligature was then placed about the portal vein. By partly closing this ligature any desired degree of increased portal resistance could be obtained. Typical kymograph records thus obtained are shown in figure 5.

From these records, it is seen that an increased portal resistance sufficient to raise the portal blood pressure 30 mm. Hg, more than three times the maximum rise during peptone shock, is practically without result on the systemic blood pressure. Even with complete ligation of the portal vein (*E*), the fall in carotid pressure is insignificant, when compared with the blood pressure fall in peptone shock. During the resulting intestinal stasis the portal blood pressure rapidly rises and becomes approximately equal to the simultaneous aortic pressure.

*2. Mechanically increased resistance to hepatic outflow.* To prepare animals for this test the venae cavae of eighteen dogs were ligated immediately below the liver. Nine of these dogs died of shock within from twelve to twenty-four hours. One dog died three days later of local thrombosis. The remaining dogs made uneventful recoveries.

The dogs showed marked cyanosis of the hind quarters for several days after the operation. Examination of the dogs six weeks later, at the time of the test, showed no cyanosis. There was marked hypertrophy of the collateral circulation,

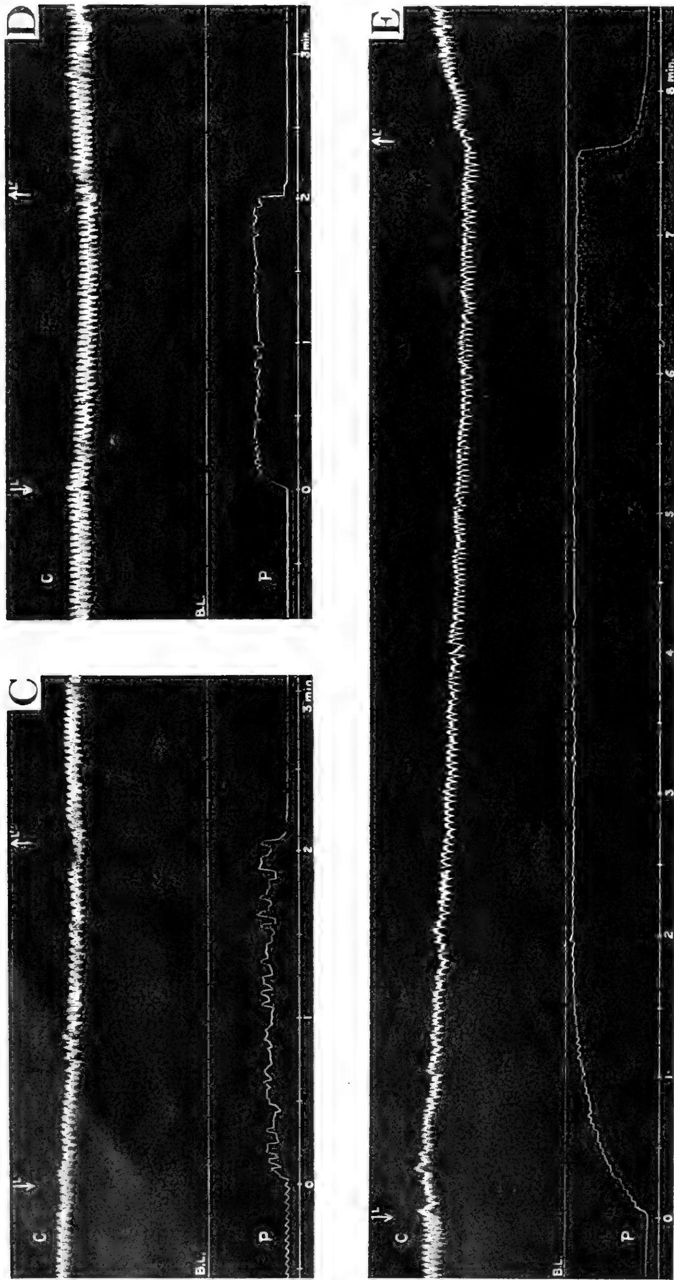


Fig. 5. Effects on Carotid Blood Pressure of Mechanically Increased Portal Resistance

*L* = Portal ligature tightened. *L'* = Portal ligature opened. *C* = Mechanical resistance sufficient to raise the portal blood pressure 16 mm. Hg. *D* = Mechanical resistance sufficient to raise the portal blood pressure 30 mm. Hg. E = Intestinal stasis.

particularly of the lumbar branches of the vena cava, the superficial abdominal veins, and the azygos veins.

To make the tests, the dogs were prepared for parallel tracings of the carotid and portal blood pressure. An unclosed ligature was then placed about the vena cava, immediately above the liver. By gradually tightening this ligature any desired degree of increased resistance to hepatic outflow could be produced, without interfering with the return circulation from the hind quarters. A typical record thus obtained is shown in figure 6.

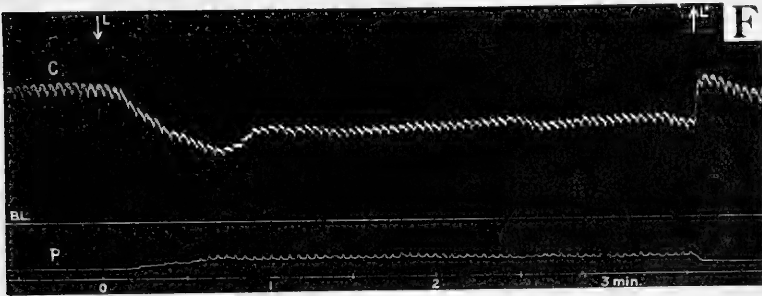


FIG. 6. EFFECTS ON CAROTID BLOOD PRESSURE OF MECHANICALLY INCREASED RESISTANCE TO HEPATIC OUTFLOW

Partial ligation of vena cava in specially prepared dogs, sufficient to raise the portal blood pressure 10 mm. Hg, the maximum rise during peptone shock. Carotid blood pressure falls from 110 mm. Hg to 60 mm. Hg by the end of forty-five seconds, but increases to 80 mm. Hg by the end of two minutes. (Corresponding carotid blood pressure during peptone shock = 30 mm. Hg.)

From this record it is seen that mechanical obstruction to hepatic outflow sufficient to raise the portal blood pressure 10 mm. Hg, which is greater than the maximum rise in portal pressure during peptone shock, is without marked effect on the systemic blood pressure. The carotid pressure usually falls from 25 mm. to 40 mm. Hg. on tightening the ligature, but almost fully recovers by the end of two minutes. It is only when the vena caval ligature is completely closed so as to produce combined hepatic-intestinal stasis, that a fall in carotid pressure is produced at all comparable with the fall during peptone shock.

Not only does carefully controlled increased resistance to hepatic outflow fail to produce the characteristic fall in carotid pressure, but it also fails to produce the characteristic local circulatory changes. The characteristic local changes in peptone shock, are a pronounced engorgement and cyanosis of the liver and intestines. Even with complete hepatic-intestinal stasis, this marked cyanosis is not produced. The color remains semi-arterial even after ten minutes' complete interruption to local blood flow. The marked local cyanosis, therefore, can not be regarded as a result of increased hepatic resistance. It is presumably due to increased functional activity of the local tissues.

## CONCLUSION

We, therefore, concluded that increased resistance to hepatic blood flow cannot be regarded as a major factor in peptone shock. Theories of peptone shock emphasizing this factor are therefore no longer tenable.

## SUMMARY

1. The portal blood pressure increases about 7 mm. Hg during the first forty-five seconds of peptone shock. The pressure then gradually falls, reaching normal in from eight to twelve minutes.

2. Perfusions of isolated livers show an increased resistance to perfusion flow as a result of the action of peptone or of peptone-blood mixtures. The resistance reaches a maximum during the second minute, and then gradually decreases, being restored to approximately normal by the tenth minute.

3. Mechanically increased resistance to hepatic outflow sufficient to raise the portal blood pressure 10 mm. Hg, the maximum rise during peptone shock, does not cause the hepatic-intestinal cyanosis characteristic of the peptone reaction, nor the characteristic fall in carotid blood pressure.

4. Theories of peptone shock based solely on the hepatic mechanical factor are, therefore, no longer tenable.

## REFERENCE

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## III. EXTRA-HEPATIC MECHANICAL REACTIONS IN PEPTONE SHOCK

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In a previous paper (1) it was shown that unneutralized Witte's peptone produces mechanical reactions in the isolated canine liver identical with the hepatic mechanical reactions in peptone shock. It was further shown that these hepatic reactions are in themselves insufficient to account for the clinical picture of peptone shock. We have therefore studied possible mechanical reactions in other tissues. The extra-hepatic parts thus far tested are: the isolated lungs, the isolated intestines, the isolated hind quarters, and the isolated heart. The reactions were studied by perfusion methods.

### MECHANICAL REACTIONS IN THE ISOLATED CANINE LIVER

To make the hepatic test, the inferior vena cava is ligated immediately below the liver; an afferent cannula is tied in the portal vein; the hepatic artery is ligated; and an efferent cannula placed in the vena cava immediately above the diaphragm. The liver is now perfused with Ringer's solution, followed, without change of pressure, with Ringer's solution containing varying amounts of peptone. Temperature, 38°C.; perfusion pressure, 20 mm. Hg.

*1. Reactions with different peptones.* As pointed out in our previous paper, commercial peptones differ markedly in their reactions with the isolated liver. Equally marked differences are produced by varying the reaction of the perfusion fluid. Typical data showing these differences are recorded in figure 1.

From these data it is seen that unneutralized (alkaline) Witte's peptone A (a pre-war product) produces hepatic mechanical

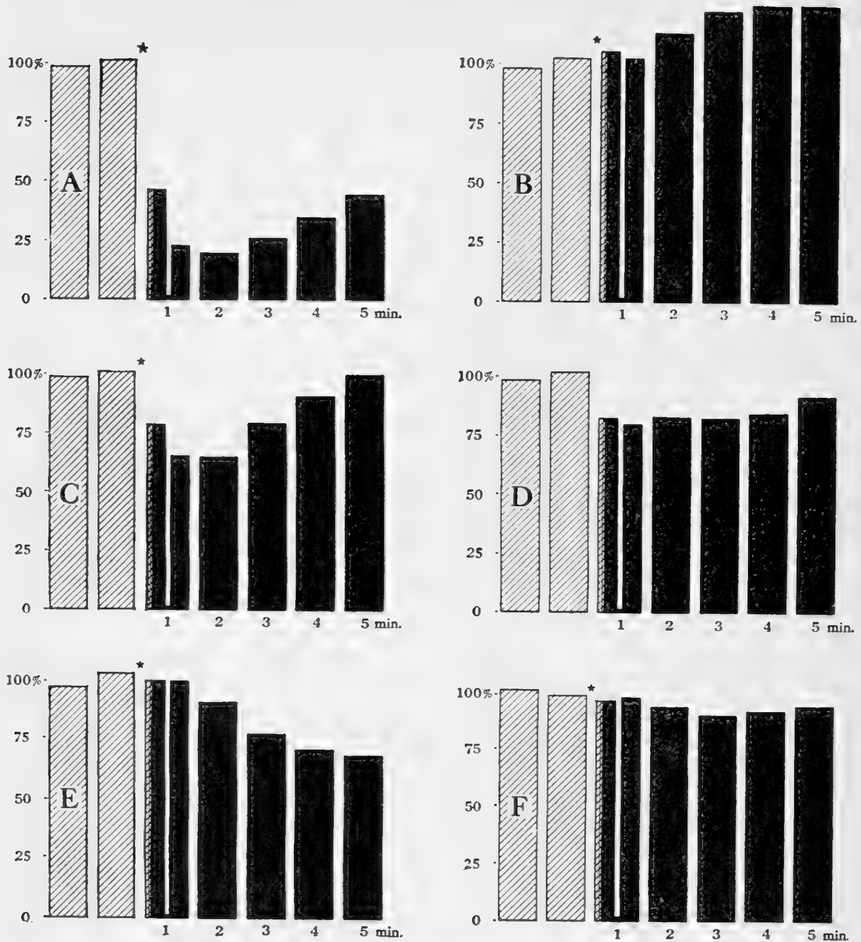


FIG. 1. HEPATIC MECHANICAL REACTIONS WITH DIFFERENT PEPTONES

Cross-hatched areas show perfusion rate per minute with Ringer's solution. Black areas show rate per minute (or half-minute) with Ringer's solution containing peptone. Star (*) shows time of changing perfusion clamps.

$A$ = 1.3 per cent unneutralized Witte's peptone A.
$B$ = 1.3 per cent neutralized Witte's peptone A.
$C$ = 1.3 per cent unneutralized Witte's peptone B.
$D$ = 1.3 per cent Witte's peptone B, plus $NaHCO_3$.
$E$ = 1.3 per cent peptone "Difco."
$F$ = 1.3 per cent peptone "Difco" plus $NaHCO_3$.

Reactions of perfusion fluids: $A$, $D$, and $F$ = 0.05 per cent $NaHCO_3$; $C$ = 0.035 per cent $NaHCO_3$; $E$ = 0.03 per cent $NaHCO_3$; $B$ = 0.015 per cent $NaHCO_3$.

reactions sufficient to reduce the rate of perfusion flow 80 per cent by the end of one minute. On neutralizing this peptone, it completely loses its power to produce this reaction. Unneutralized (faintly alkaline) Witte's peptone B (a post-war product) produces less marked hepatic reactions. The reactions with this peptone are greatly decreased, by increasing its alkalinity to that of peptone A. Peptone "Difco" gives very sluggish

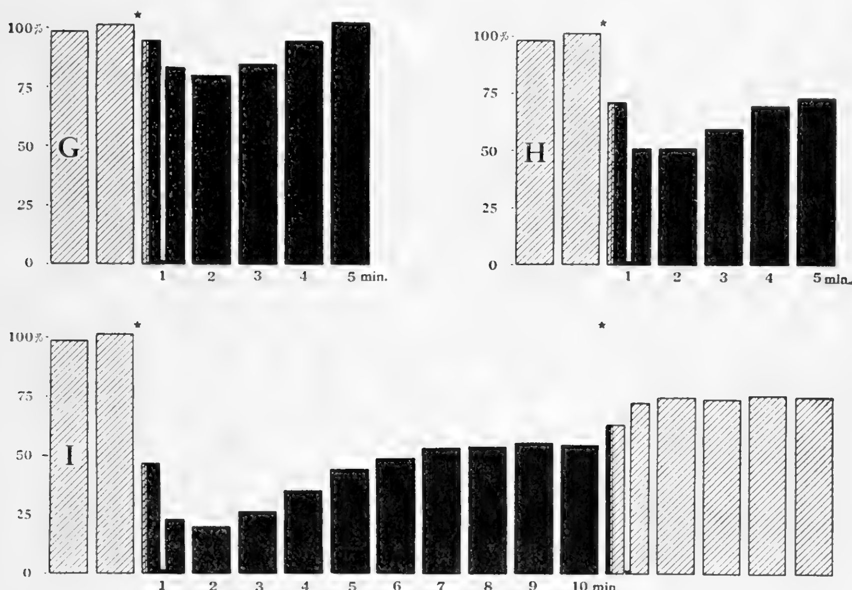


Fig. 2. Effects of Varying the Peptone Concentration

Hepatic perfusions

$G$ = 0.5 per cent unneutralized Witte's peptone A
$H$ = 0.9 per cent unneutralized Witte's peptone A
$I$ = 1.3 per cent unneutralized Witte's peptone A

reactions with the isolated liver. Its reactions are practically lost by increasing its alkalinity to that of peptone A.

Unneutralized Witte's peptone A was selected for the routine tests. All tests hereinafter recorded are with this peptone.

*2. Reactions with different peptone concentrations.* Marked differences in the hepatic mechanical reactions are produced by varying the peptone concentration of the perfusion fluid. Typical data are recorded in figure 2.

From these data it is seen that 0.5 per cent Witte's peptone A produces mechanical reactions sufficient to reduce the perfusion flow about 25 per cent by the end of one minute, a reaction from which the liver completely recovers by the end of four minutes. With 0.9 per cent Witte's peptone A, there is a reduction of 50 per cent in the perfusion rate by the end of one-half minute, with only partial recovery by the end of five minutes. With 1.3 per cent Witte's peptone A there is a reduction of 80 per cent by the end of one-half minute, with only slight recovery by the end of ten minutes. In all of our tests the maximum reaction was observed during the second minute. Recovery always began during the third minute.

### MECHANICAL REACTIONS IN THE ISOLATED CANINE LUNGS

To make the pulmonary test, the inferior vena cava, azygos vein, and aorta are ligated immediately adjacent to the heart, an afferent cannula is tied in the superior vena cava, and an efferent cannula in the left auricular appendage. The lungs are then inflated to their midrespiratory volume. Perfusion pressure, 25 mm. Hg. to 30 mm. Hg. Typical data are recorded in figure 3.

In all of our tests, the mechanical reactions in the isolated lungs were more pronounced than the hepatic mechanical reactions with the same peptone concentration. Thus, 0.25 per cent Witte's peptone A, reduces the rate of pulmonary perfusion 33 per cent by the end of one minute, increasing to a 40 per cent reduction by the end of five minutes. With 0.5 per cent Witte's peptone A, the minimum peptone concentration producing distinct hepatic reactions, the rate of pulmonary perfusion is reduced 70 per cent by the end of one minute, with no tendency to recovery by the end of five minutes. With 1.3 per cent Witte's peptone A, the pulmonary rate is reduced 80 per cent during the second minute, with almost no recovery.

It is interesting to note, that a second pulmonary perfusion with 1.3 per cent Witte's peptone A, after a five minute's intermediary perfusion with Ringer's solution, gives almost no secondary reaction. A similar refractory period following an initial peptone perfusion was observed with the isolated liver.

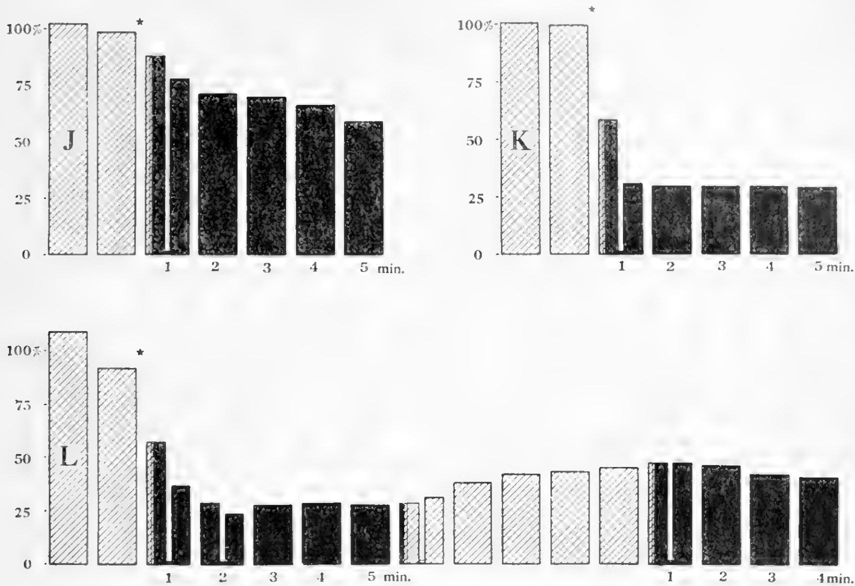


Fig. 3. Mechanical Reactions in the Isolated Canine Lungs

$J$ = 0.25 per cent unneutralized Witte's peptone A
$K$ = 0.5 per cent unneutralized Witte's peptone A
L = 1.3 per cent unneutralized Witte's peptone A

## MECHANICAL REACTIONS IN THE ISOLATED CANINE INTESTINES

To make the intestinal test, the descending colon is ligated immediately above the entrance of the inferior mesenteric artery; the duodenum is ligated close to the pylorus; ligatures are placed about the splenic blood vessels; an afferent cannula is tied in the aorta immediately below the superior mesenteric artery, and an efferent cannula in the portal vein. Perfusion pressure, 80 mm. Hg. to 100 mm. Hg. Typical data are recorded in figure 4.

In none of our intestinal tests was there a slightest suggestion of an increased perfusion resistance as a result of peptone action. There was uniformly a slight increase in perfusion flow, reaching a maximum during the second minute, usually with partial recovery by the end of five minutes.

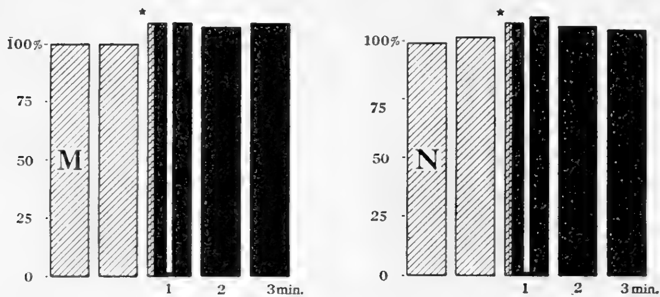


FIG. 4. MECHANICAL REACTIONS IN THE ISOLATED CANINE INTESTINES

*M* = 0.5 per cent unneutralized Witte's peptone A
*N* = 1.3 per cent unneutralized Witte's peptone A

MECHANICAL REACTIONS IN THE ISOLATED CANINE HIND QUARTERS

To make this test, an afferent cannula is placed in the abdominal aorta, and an efferent cannula in the abdominal vena cava. The collateral circulation is controlled by well padded steel bars, curved to fit over the lumbar muscles, and held in place by set screws. Perfusion fluid, Locke's solution containing 0.5 per cent filtered, defibrinated blood from the same animal. Perfusion pressure, 80 mm. Hg. to 110 mm. Hg.

The canine hind quarters, isolated by this method, usually show a marked reduction in vascular tone, on control perfusion with Locke's solution. The vascular tone, however, is never completely lost, since a distinct increase in perfusion flow is produced by the addition of amyl nitrite to the perfusion fluid. The isolated hind quarters react vigorously to adrenalin and to barium chloride. In a third of our cases, the vascular tone was fairly well preserved by this method, at least during the initial stages of the perfusion. Typical tests are shown in figure 5.

In none of our tests was there the slightest suggestion of an increased perfusion resistance as the result of peptone action. There was uniformly an increased perfusion flow, varying from a 10 per cent increase in the vascular atonic hind quarters, to a

150 per cent increase in the vascular tonic hind quarters. At the height of the peptone reaction the rate of flow was identical with the perfusion rate with amyl nitrite.

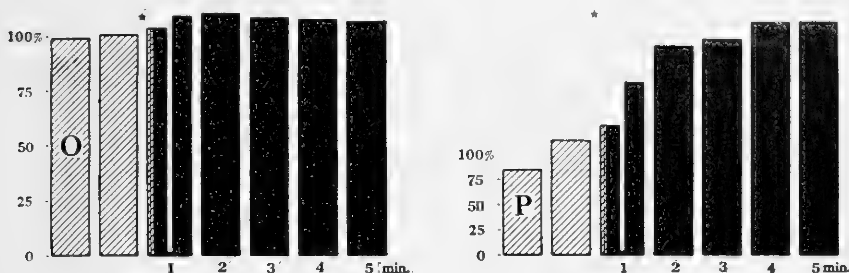


FIG. 5. MECHANICAL REACTIONS IN THE ISOLATED CANINE HIND QUARTERS

*O* = vascular atonic hind quarters; 1.3 per cent unneutralized Witte's peptone A.

*P* = vascular tonic hind quarters; 0.9 per cent unneutralized Witte's peptone A.

## MECHANICAL REACTIONS IN THE ISOLATED CANINE HEART

The heart was perfused with well aerated Locke's solution containing 0.5 per cent filtered, defibrinated blood, by the technique described in a previous paper (2). Perfusion pressure, 80 mm. Hg. Typical data are shown in figure 6.

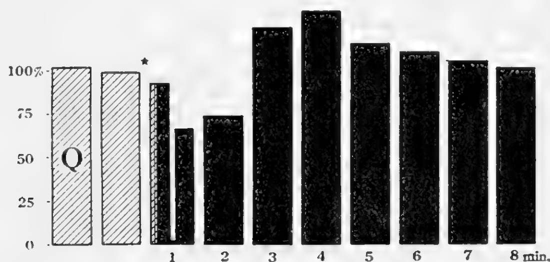


FIG. 6. MECHANICAL REACTIONS IN THE ISOLATED CANINE HEART

*Q* = 1.3 per cent unneutralized Witte's peptone A

From these data it is seen that the cardiac reactions stand intermediary between the hepatic reactions and the reactions in the hind quarters. On the addition of peptone there is an

initial increase in perfusion resistance, reaching its maximum by the end of one minute. This is succeeded by a decreased perfusion resistance, reaching its maximum by the end of three minutes, with full recovery by the end of eight minutes. The cardiac reactions, however, are never sufficient to alter the perfusion rate more than 25 per cent.

## CONCLUSIONS

Assuming that the data from peptone perfusions with isolated organs can be carried over unchanged to the peptone reactions in the intact animal, we therefore conclude that the main mechanical factors operative in peptone shock in dogs are:

1. A marked increase in resistance to blood flow through the liver, reaching a maximum by the end of one minute, with partial or complete recovery by the end of eight minutes.

2. An even more pronounced increase in resistance to blood flow through the lungs, with practically no tendency to recovery by the end of eight minutes.

3. A decreased resistance to blood flow through the intestines, skin, and skeletal muscle, reaching a maximum by the end of one minute, with only partial recovery by the end of eight minutes.

The combined effect of these mechanical factors conceivably accounts for the characteristic fall in arterial blood pressure during peptone shock.

The histological mechanism of the altered perfusion resistance in these organs will be reported in a subsequent paper.

## SUMMARY

1. Unneutralized Witte's peptone, tested by perfusion methods on isolated canine tissues, gives mechanical reactions in the following parts:

*a. Isolated liver:* Marked increase in perfusion resistance, reaching its maximum by the end of one minute, with partial or complete recovery by the end of eight minutes.

*b. Isolated lungs:* Reactions similar to those in the isolated

liver. The reactions, however, are more pronounced, with practically no tendency to recovery by the end of eight minutes.

*c. Isolated intestines:* Slight decrease in perfusion resistance, reaching its maximum by the end of one minute, with partial recovery by the end of eight minutes.

*d. Isolated hind quarters:* Reactions similar to these in the isolated intestines, but with less tendency to recovery by the end of eight minutes.

*e. Isolated heart:* Reactions intermediary between those in the isolated liver and the isolated hind quarters.

2. The combined effect of these mechanical reactions is conceivably sufficient to account for the characteristic clinical picture of peptone shock.

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# BACTERIAL ANAPHYLAXIS[1]

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In 1900 Lustig and Galeotte (1) apparently demonstrated that clinical anaphylaxis could be produced in experimental animals using bacterial proteins. They worked with nucleo-proteins from *B. pestis* and *Microspira comma* prepared by the Wooldridge method and the use of strong KOH to dissolve the cell membranes. Their results were confirmed by many workers among whom were, Del Conte, Heller, DeBonis, Tiberti, Savagnone. The European work up to 1913 is well summarized by Lustig (1). In America Rosenau and Anderson (2) were pioneers in the field of bacterial anaphylaxis.

Two sources of error have entered into much of the work reported, namely, methods of preparation of protein which racemized (3) it and the use of the intravenous method of demonstration which has caused to be included anaphylactoid (4) as well as true anaphylactic reactions. However, because of the vast amount of positive results obtained, it seems to be well established that suitable laboratory animals can be sensitized with bacterial protein and that when sensitized they give the characteristic clinical picture as a result of a test injection. No work seems to have been done making use of the smooth muscle reaction devised by Schultz (5) and Dale (6). This reaction has more recently been considered by Wells (7) as important evidence of sensitization. It was thought that if this method was applicable to bacterial proteins it might clear up many points of

[1] This work was partially supported by appropriations made by the Research Committee of the University of Kansas.

disputed relationships and be of fundamental value in classifying bacteria in addition to throwing more light upon the antigenic properties of nucleo-proteins.

The work of this paper is a continuation of our preliminary report (8) and includes sensitization with bacterial proteins of *B. typhosus* and *B. enteritidis* that were not racemized and the testing out for sensitization by intraperitoneal test injections, passive sensitization and also by the smooth muscle reaction of Schultz and Dale. Preliminary to this, experiments were conducted to determine the effect and toxic dose of bacterial protein for excised intestinal loops of 20 normal rabbits and 20 guinea-pigs and for excised uterine horns of 10 young virgin guinea-pigs. In addition to this normal controls were run parallel with the test experiments.

The following methods of preparation of bacterial protein were tried out in the course of the investigation:

1. Vaccines of *Bacillus typhosus, paratyphosus "A"* and *"B"* and *B. enteritidis* were made by washing off the growth from agar plates or Kolle's flasks with sterile physiological salt solution and the organisms killed by heat.

2. Old broth cultures of the same organisms were used since they would contain bacterial protein in solution.

3. Autolysed extracts of these organisms prepared by washing off the growths from agar plates or Kolle's flasks with distilled water and permitting to autolyze at ice box temperature.

4. Concentrated bacterial extracts were obtained by growing the bacteria in large pans similar to the one devised and described by Vaughan (9). The surface growth was washed off with a minimum amount of sterile physiological salt solution and aspirated into a sterile flask. Bacterial counts were made using a hemocytometer and dilute fuchsin as a diuting fluid after the growth had been washed by repeated centrifuging at high speed and the volume determined. It was then concentrated to a minimum volume by centrifuging at high speed. We will call this original concentrated suspension "O." Three different methods were tried in attempting to prepare a satisfactory extract. These methods are as follows:

*Method A.* One part of the original concentrated suspension "O" was mixed with 9 parts of $\frac{N}{100}$ NaOH. The NaOH was added slowly to

the bacterial protein and ground in a mortar for thirty minutes. This was allowed to extract at room temperature for six hours and in the cold for varying lengths of time ranging from twelve hours to twenty-four hours, shaking a number of times in the interval. This was then neutralized to phenolphthalein with $\frac{N}{100}$ acetic acid and kept frozen until used. It was thought that using dilute sodium hydroxide and extracting in the cold would prevent racemizing the protein and that after neutralization the sodium acetate would function as a buffer. Ethyl alcohol was used to precipitate part of this and the precipitate taken up in a minimum amount of normal, salt solution.

*Method B.* To 1 part of suspension "O" was added 8 per cent sodium bicarbonate in proportion of 1 part of bacterial suspension to 9 parts of bicarbonate. This was heated to 55°C. for one hour and centrifuged and the bacterial protein was precipitated from the supernatant fluid with ethyl alcohol in proportion of 9 parts of alcohol to 1 part supernatant fluid or extract. The precipitate was taken up in distilled water, just enough to take up the soda bicarbonate in solution. This was neutralized to phenolphthalein with HCl, just enough to discharge the color. This was again centrifuged and the superantant fluid was saved as the bacterial extract. This was kept frozen in brine until used.

*Method C.* The most satisfactory method was as follows: One cubic centimeter of suspension "O" was put in each of 10 test tubes. In tube 1, 9 cc. of $\frac{N}{1}$ NaOH was added, to tube 2, 8 cc. of $\frac{N}{1}$ NaOH and 1 cc. of physiological salt solution; tube 3, 7 cc. of $\frac{N}{1}$ NaOH and 2 cc. of physiological salt solution; the amount of $\frac{N}{1}$ NaOH was thus decreased until in tube 10, 9 cc. of physiological salt solution alone was added. These tubes were shaken for one hour at room temperature. In this way the least amount of $\frac{N}{1}$ NaOH was determined that would give a fairly clear opalescent solution of the bacterial protein. The proper amount of $\frac{N}{1}$ NaOH was then added to a measured amount of bacterial suspension and allowed to incubate for one hour at room temperature with continuous agitation. It is a well known fact that shaking is a fair substitute for heat in so far as extraction is concerned. The extract was then nearly neutralized to phenolphthalein with $\frac{N}{1}$ HCl and finally neutralized with $\frac{N}{1}$ HCl. This gave a solution of bacterial protein in approximately normal salt solution representing a count of 75 billion bacteria per cubic centimeter. This was kept frozen in brine until used.

The technic used by us to study the smooth muscle reaction in both normal and sensitized animals is described in another communication (10). It is essentially that of Schultz and Dale except that Tyrode's solution is used instead of Ringer's or Locke's. Young virgin guinea-pigs weighing approximately 240 grams were used when excised uterine horns were to be studied. For intestinal loops guinea-pigs weighing from 240 to 300 grams were used as were also rabbits weighing from 1500 to 2000 grams. Several intestinal loops were obtained from each animal, two were set up immediately, the remainder were suspended in Tyrode solution and kept at 38°C. for additional experiments. These would remain active for several hours if they were kept in slight tension by a small weight attached at the lower end. In this way test material consisting of samples of bacterial protein prepared in various ways could be tried out. The results obtained in our other work upon anaphylaxis (10) abundantly demonstrates the satisfactory nature of the technic.

## THE EFFECT OF BACTERIAL SUSPENSION AND EXTRACTS UPON INTESTINAL LOOPS AND UTERINE HORNS OF NORMAL ANIMALS

In this paper we are confining ourselves almost exclusively to a study of the antigenic value of typhoid protein although we have included some data for *B. paratyphosus "B,"* and *B. enteritidis*. The toxic effect of several strains of *B. suipestifer*, *B. avisepticus*, *B. coli communis*, *B. coli aerogenes*, *Microspira-comma*, *B. subtilis*, *B. diphtheriae*, and *Staphylococcus aureus* were studied.

The apparent toxic effect of the bacterial protein on normal excised intestinal loops and uterine horns may not be, and probably is not, due to the protein molecule since we have shown that purified crystalline egg albumin is not toxic (10). We have found that slight traces of ammonium or magnesium sulphate cause marked contraction and it is well known that histamine and perhaps many other substances will produce a smilar result. We found that when we attempted to purify our bacterial proteins by

precipitation with alcohol it became exceedingly toxic. This we might expect in view of Vaughan's (9) work on bacterial split products. In preparing our suspensions of bacterial protein we made use of centrifuging at high speed and washing with sterile physiological salt solution to get rid of as much of the products of growth as possible. The concentrated protein prepared as in 4 C was only slightly toxic when injected intraperitoneally into normal guinea-pigs; i.e., 10 cc. of typhoid protein were tolerated by each of 9 normal pigs without any marked reactions or death of the animal, 18 cc. produced death in 2 out of 3 normal pigs in twenty-four hours. We also exercised great care in removing the bacterial growth from the surface of the agar; we found that 3 per cent agar also rendered this more feasible.

With the excised loops and uterine horns a dose of more than 1 cc. of a 1:10 dilution of bacterial protein prepared by methods 1, 2, and 3 usually gave a toxic reaction, this did not occur with 0.5 cc. or less. Bacterial protein prepared by method 4 C was the least toxic to smooth muscle considering its concentration. The reacting or toxic dose was approximately 1 cc. of straight typhoid protein. When washings from twenty-four-hour agar plates were used we could frequently use as much as 0.8 cc. and sometimes 1 cc. The toxic effect of native proteins is customarily thought of and described as giving a tracing similar to the specific response and differing in that it lacks specificity and can be repeated on the same loop or uterine horn, i.e., desensitization does not result. This type of response is well illustrated in figures 1, 2, and 3 which show the effect upon intestinal loops of normal guinea-pigs and rabbits, respectively.

## EFFECT OF BACTERIAL PROTEIN UPON INTESTINAL LOOPS AND UTERINE HORNS OF ACTIVELY AND PASSIVELY SENSITIZED ANIMALS

We immunized 75 guinea-pigs with vaccines of *Bacillus typhosus* and *Bacillus enteritides* and varied the number of injections from 1 to 13. The dosage was approximately five hundred million organisms. The time interval between injections was from three to five days. The time intervals after the

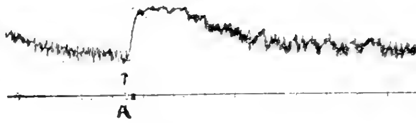


FIG. 1

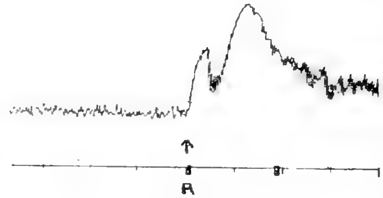


FIG. 2

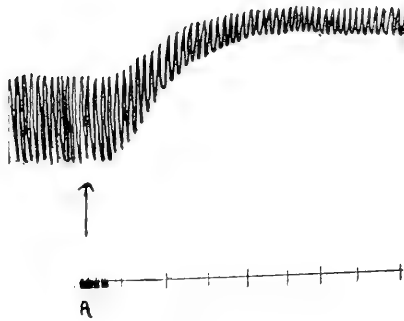


FIG. 3

FIG. 4

FIG. 5

FIG. 1. Intestinal loop (1), normal guinea-pig "B," series "N." Enteritidis suspension added at "A" showing toxic effect of large doses.

FIG. 2. Intestinal loop (1), normal guinea-pig "B," series "N." *Staphylococcus aureus* suspension added at "A" showing toxic effect of large doses.

FIG. 3. Intestinal loop (1) of normal rabbit 94. Autolysed suspension of *Bacillus enteritidis* added at "A" showing toxic effect of large doses.

FIG. 4. Intestinal loop from guinea-pig 85 sensitized to *Bacillus typhosus* protein. One cubic centimeter protein of *Bacillus typhosus* added at "A."

FIG. 5. Left uterine horn from virgin guinea-pig sensitized to typhoid protein. One-half cubic centimeter protein added at "A."

FIG. 6 A

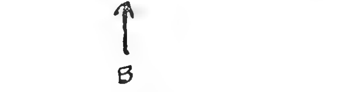


FIG. 6 B

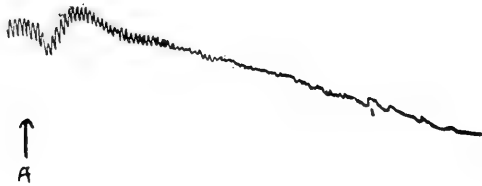


FIG. 7 A

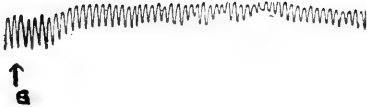


FIG. 7 B

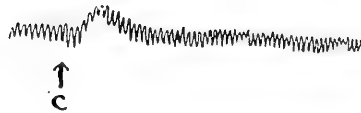


FIG. 7 C

FIG. 6 A. Intestinal loop from guinea-pig 81 vaccinated against *Bacillus enteritidis*. Nontoxic dose of enteritidis suspension added at "A." This shows the only apparent specific response obtained.

FIG. 6 B. Same loop as in 6 A after change of Tyrode solution. One cubic centimeter of same suspension added at "B" with no response.

FIG. 7 A. Intestinal loop from rabbit immunized against *Bacillus enteritidis*. Suspension added at "A" shows stimulation of inhibitory mechanism.

FIG. 7 A and B. Same loop as in 7 A after change of Tyrode solution showing that suspensions of suipestifer and avisepticus had no effect when added at "B" and "C," respectively.

last injection were studied and varied from eight to thirty days. In all but one instance bacterial proteins did not produce the specific smooth muscle contraction in intestinal loops or uterine horns. Figures 4 and 5 are typical of these results for intestinal loops and uterine horns from guinea-pigs immunized against bacterial protein used by us. Figures 6, *A* and *B* show the one apparent specific response given by an intestinal loop. The curve is not what one would call a typical one such as is obtained with soluble proteins. Old broth cultures and autolyzed cultures gave correspondingly negative results when used either for immunization or as test material. When toxic doses were used the loops from these immunized animals seemed to have about the same threshold for stimulation as normals but on the whole, responded with greater intensity than normals.

In addition to this, 14 rabbits were immunized with these vaccines until the agglutinin titer of their sera was from 1:1000 to 1:8000. The smooth muscle reaction using intestinal loops was not obtained in a single instance.

The immunization experiments with concentrated extracts of bacterial protein gave uniformly similar results with the intestinal loops and uterine horns of 92 virgin guinea-pigs sensitized with concentrated proteins prepared by methods 4 A, B, and C, respectively. Of this number 52 were sensitized with protein prepared by the later method. In order to determine whether the animals were sensitized we resorted to passive immunization. The passively sensitized pigs were given a test injection after twenty-four hours, using a dose that was well tolerated by normal guinea-pigs. For typhoid protein prepared by the method 4 C, 10 cc. represented such a dose. We found, however, that 8 cc. were sufficient to produce protracted anaphylactic shock and death within two and one-half hours in passively or actively sensitized guinea-pigs when given intraperitoneally. Another method which we resorted to was that of actively immunizing rabbits with this concentrated typhoid protein until their sera gave a good precipitin reaction with the bacterial proteins. Six guinea-pigs were then passively sensitized with immune rabbit serum. Three were tried out for clinical anaphylaxis and others were

used for smooth muscle work. Again clinical anaphylaxis was produced with negative results for the smooth muscle reaction using both uterine horns and intestinal loops. We found by the clinical test that 1 injection of bacterial protein prepared as in 4 C did not sensitize but that 3 or more injections gave quite consistent results. The initial injection was 2 cc. followed with injections of 5 cc. at five-day intervals.

In the course of our work we have noted that occasionally the inhibitory mechanism of the intestinal loop or uterine horn is stimulated by certain bacterial suspensions or extracts. This we first noted in the intestinal loop of rabbit 1. This animal had been immunized against *Bacillus enteritidis.* When a reacting dose of a suspension of *Bacillus enteritidis* was added a drop in tone and loss of contractility occurred which was restored upon changing Tyrode solution as shown in figure 7, *A*.

A similar curve was obtained when a suspension of *Bacillus typhosus* or *Bacillus coli* was added. On the other hand when a suspension of *Bacillus suipestifer* or *Bacillus avisepticus* was added a temporary rise in tone only resulted as shown in figure 7, *B* and *C*. Human blood produced the usual rise in tone but repeated trials with the various suspensions gave results corresponding to the tracings just described.

It would seem that this might be of interest clinically since many commercial vaccines, etc., contain products of growth and are used extensively both prophylactically and therapeutically and introduced by either subcutaneous or intravenous methods. Our results would suggest that variations from the normal response might occasionally occur. It is also conceivable that a stimulation of an inhibitory mechanism in sensitized guinea pigs might account for occasional failures to produce asphyxia when a test injection is given intravenously as well as the usual explanation of lack of sensitization.

Our results then would indicate that the smooth muscle reaction is not necessarily an index of sensitization in so far as bacterial anaphylaxis is concerned. The observation that more than 1 injection is needed for sensitization and the apparent absence of smooth muscle sensitization confirms the statement of Wells (11)

that nucleo-proteins are poor antigens. It is conceivable that if the bacterial protein could be freed from all toxic substances enough might be added to the bath to get a specific reaction but this we doubt in view of the high concentration we were able to use. We are investigating the antigenic value of other species of bacteria and also of beta nucleo-proteins similar to those reported upon by Wells (11).

## CONCLUSIONS

Active or passive sensitization against bacterial protein of *Bacillus typhosus*, *para-typhosus B* or *Bacillus enteritidis* is not demonstrable by the smooth muscle reaction of Schultz and Dale.

In a small percentage of animals, bacterial suspensions of *Bacillus typhosus*, *Bacillus enteritidis*, *Bacillus para-typhosus "B,"* and *Bacillus coli* stimulated the inhibitory mechanism of intestinal loops or uterine horns. In the rabbit this is evidenced by a drop in tone and loss of contractility while in the guinea-pig the drop in tone is the noticeable observation. Suspensions of other organisms and of soluble proteins either stimulated the accelerating mechanism or had no effect.

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# OBSERVATIONS ON A SUBSTANCE IN IMMUNE HORSE SERUM WHICH INTERFERES WITH ALEXIN FIXATION

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About a year ago one of the writers published a paper on the essential identity of antibodies. The conception expressed in this paper postulated that every individual antigenic substance aroused in the properly treated animal the formation of a single specific antibody. When this antibody united with its antigen, the latter was altered or sensitized. The several effects spoken of as agglutination, precipitation, sensitization to alexin, opsonization, etc., were regarded merely as indirect methods of observing this union, dependent for their occurrence upon the physical state of the antigen, and upon environmental factors, such as the presence of electrolytes, viscosity, inactive protein admixtures (in their effects upon colloidal behavior), the presence of alexin or of leucocytes, etc. This point of view was suggested to us by some observations we made upon precipitin reactions in 1912, and expressed, in a tentative way, in a paper published at that time. It apparently had occurred to Bail and Hoke as early as 1908, and was very definitely formulated by Dean in a lecture delivered by him in 1917 which, unfortunately, we had not seen when our paper was published.

One of the most important objections brought forward against this "unitarian" conception of antibodies is the difficulty frequently encountered in attempts to demonstrate quantitative parallelism for the various antibody effects in one and the same serum. Of still greater weight in refuting this point is the fact that occasionally an antiserum found quite potent in some of

its antibody effects, may appear to be entirely lacking in another. To some extent we think we have answered objections of this kind in the paper referred to above, where, in our final summary, we admitted that it might be impossible at the present time to bring absolute proof for our conception, but that we wished simply to state it, believing that sufficient evidence had been adduced to throw the "burden of proof upon those who still cling to the separation, and the conception of a separate structure for agglutinins, precipitins, bacteriolysins, etc."

In the course of the last two years we have been engaged in studying bacterial hypersensitiveness, especially as manifested in such phenomena as the tuberculin reaction, and in continuing this work we were led to give considerable attention to an apparently non-protein residue obtained from our bacterial extracts, which gave specific and sharp antibody reactions with homologous sera. Such substances were obtained from a variety of bacteria (staphylococci, pneumococci, influenza bacilli, tubercle bacilli), were antigenic in the sense that they were specifically precipitable, and when sensitized showed specific alexin fixation. Curiously enough, however, they have so far completely resisted all our attempts to induce antibody formation by injecting them into animals. This work is now in press and will appear before the present paper is published. While carrying out these experiments, however, we encountered certain irregularities in the behavior of one of these antigens, the pneumococcus "residue," which seemed to us to have so important a bearing upon our conception of the identity of all antibodies, that they distinctly called for study in this direction. It is the purpose of this paper briefly to present these studies.

The so-called "residue" antigens with which we are working, are made by the extraction of bacteria, grown upon large agar plates, in slightly alkaline salt solution, the subsequent removal of materials precipitable with acid in the cold, followed by further removal of proteins by boiling with acid, filtration and neutralization. Detailed description of the process is found in the preceding paper referred to above, and is omitted because it has no important bearing upon the work here recorded. This clear

"residue" antigen is possibly protein-free, but this point is being studied in considerable detail at the present time. Its chemical nature is naturally not easy to determine. It is a water-clear fluid which gives only very slight precipitate on the addition of ten or more volumes of absolute alcohol, is powerfully and specifically precipitated with homologous sera, and in most cases gives rise to the complete fixation of two units of alexin in quantities often as low as 0.01 to 0.001 cc., when sensitized with specific immune serum. Such precipitation and alexin fixation was easily demonstrable with all the bacterial residues except that obtained from the Type I pneumococcus, the only representative of the pneumococcus group with which we worked. This pneumococcus residue invariably gave powerful precipitation, but as tested with several varieties of antipneumococcus horse-serum and a bovine antipneumococcus serum supplied us by Dr. Huntoon, failed completely to show alexin fixation. It seemed, therefore, as though we might be dealing with sera in which precipitins were present, but which were entirely devoid of alexin fixing antibodies, a fact which would favor the view originally expressed by Neufeld and his coworkers, and almost universally adopted, that in alexin fixation a new antibody (Bordetscher Antikörper) were involved. In consequence, during the summer we decided to sift this matter more thoroughly and proceeded by first producing a rabbit antipneumococcus serum in the usual manner. The two sera marked serum 445 and serum 153, were the two rabbit sera used in the experiments, and these sera, as well as the horse sera employed, were found to precipitate powerfully the pneumococcus "residue" antigen. The horse serum employed consisted of two lots of antipneumococcus serum kindly supplied to us by Miss Kirkbride of the New York State Hygienic Laboratory.

Our first thought was that perhaps the pneumococcus horse sera might contain conglutinins of sufficient strength to so increase the hemolytic properties of the alexin that any moderate degree of fixing properties on the part of the sensitized antigen would be masked. In order not to prolong this paper unnecessarily, we may state that while the horse serum did seem to con-

tain a small amount of conglutinin, this could be definitely ruled out as determining the nature of our reactions.

The first experiment which follows is merely given in order to reassert the problem we were facing.

*Experiment I*

| PNEUMOCOCCUS RESIDUE ANTIGEN | | AMOUNT OF SERUM | | | FIX.TION |
|---|---|---|---|---|---|
| cc. | | cc. | | | |
| 0.5 | Antipneumococcus rabbit serum 446 | 0.05 | Alexin | Water bath and subsequent addition of sensitized red cells as usual | ++++ |
| 0.1 | Antipneumococcus rabbit serum 446 | 0.05 | Alexin | | ++++ |
| 0.01 | Antipneumococcus rabbit serum 446 | 0.05 | Alexin | | ++++ |
| 0.5 | Antipneumococcus rabbit serum 153 | 0.05 | Alexin | | ++++ |
| 0.1 | Antipneumococcus rabbit serum 153 | 0.05 | Alexin | | ++++ |
| 0.01 | Antipneumococcus rabbit serum 153 | 0.05 | Alexin | | ++++ |
| 0.5 | Normal rabbit serum (1) | 0.05 | Alexin | | ± |
| 0.1 | Normal rabbit serum (1) | 0.05 | Alexin | | – |
| 0.01 | Normal rabbit serum (1) | 0.05 | Alexin | | – |
| 0.5 | Normal rabbit serum (2) | 0.05 | Alexin | | – |
| 0.1 | Normal rabbit serum (2) | 0.05 | Alexin | | – |
| 0.01 | Normal rabbit serum (2) | 0.05 | Alexin | | – |
| 0.5 | Horse antipneumococcus serum | 0.05 | Alexin | | – |
| 0.1 | Horse antipneumococcus serum | 0.05 | Alexin | | – |
| 0.01 | Horse antipneumococcus serum | 0.05 | Alexin | | – |

This experiment, then, shows that although the horse antipneumococcus serum, as well as both the antipneumococcus rabbit sera gave powerful precipitation with the pneumococcus "residue" antigen, fixation was obtained only with the two rabbit sera; the horse sera, like the normal rabbit sera, giving rise to no fixation whatever; and this, in spite of the fact that the antipneumococcus horse serum precipitated more powerfully and more promptly than did either of the rabbit sera, and gave a more powerful agglutinating effect when mixed with whole pneumococci.

Upon further study of this irregularity, a very peculiar fact became apparent. This is illustrated in Experiment II, an-

example of a considerable number of experiments in all of which the results were consistent. It appeared that if pneumococcus "residue" antigens were sensitized with antipneumococcus rabbit serum, and antipneumococcus horse serum was then added to the sensitized antigen, in various amounts, ranging from 0.1 to 0.01 cc., and left in contact with this rabbit-serum sensitized antigen for five to ten minutes at room temperature, the fixation, which normally would have taken place had the horse serum been omitted, was prevented, the tubes going on to complete hemolysis. This is illustrated in tubes 2, 3, and 4 of Experiment II. If, on the other hand, alexin was first added to the antigen sensitized with the specific rabbit serum, and after 45 minutes contact with the alexin, horse serum was added to the complex, no such interference with fixation occurred. This is illustrated in tubes 5 and 6 of Experiment II. Tube 1 of this experiment is merely a control, reasserting a brief fixation when the "residue" antigen was sensitized with the antipneumococcus rabbit serum.

*Experiment II*

| | PNEUMOCOCCUS RESIDUE ANTIGEN | ANTIPNEUMOCOCCUS RABBIT SERUM 446 | | ANTIPNEUMOCOCCUS HORSE SERUM | | ALEXIN | | ANTIPNEUMOCOCCUS HORSE SERUM | | | RESULTS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | cc. | cc. | | cc. | | | | cc. | | | |
| 1 | 0.2 | 0.05 | Thirty minutes at 37.5°C. | | Five minutes 37.5°C. | Two units to all tubes | Forty-five minutes, 37.5°C. | | Five minutes | Sensitized red blood cells | ++++ |
| 2 | 0.2 | 0.05 | | 0.1 | | | | | | | 0 |
| 3 | 0.2 | 0.05 | | 0.05 | | | | | | | 0 |
| 4 | 0.2 | 0.05 | | 0.01 | | | | | | | 0 |
| 5 | 0.2 | 0.05 | | | | | | 0.1 | | | ++++ |
| 6 | 0.2 | 0.5 | | | | | | 0.05 | | | ++++ |

++++ = alexin fixation.
0 = no fixation.

It thus appears that there is something in the horse serum which prevented the fixation of alexin even when other conditions, such as the presence of a specific sensitizer were fulfilled.

What this body is, we do not know, but since it prevents the otherwise consistent fixation of alexin by antipneumococcus-rabbit-serum, it is quite clear that fixing sensitizers may be present in the horse serum, and yet be completely masked

by this other body. From the order in which the materials must be added in order to cause such interference with fixation, it would appear that whatever this body which is present in the horse serum may be, it interferes with the union of the alexin to the sensitized antigen-antibody complex. That the alexin is not destroyed is clear from the eventual hemolysis in these tubes, as well as from additional experiments made in this direction.

It then occurred to us that it might be possible to absorb out of the horse serum this interfering body and prove that the apparent discrepancy in the precipitating and complement fixing properties of the antipneumococcus horse serum were not due to an absence of a specific fixing antibody. We thought we could accomplish this by sensitizing washed pneumococci, taking up the sediment in salt solution, then adding a small amount of pneumococcus horse serum, allowing the mixture to stand for about ten minutes and again centrifugalizing. Control tubes were set up in which a part of the sensitized pneumococcus sediment was merely emulsified in salt solution, the horse serum being omitted. Such an experiment is represented in Experiment III.

This experiment, after reaffirming (in tube 1) that the rabbit serum, together with the "residue" antigen fixes and (in tube 2) that horse serum added to the "residue" antigen so sensitized, prevents fixation, shows that supernatant fluid "A" (which represents, respectively, 0.1 and 0.05 cc. absorbed horse serum) no longer prevents fixation by the rabbit serum antigen complex. The experiment is a complete success insofar as it shows that the interfering body can be absorbed out of the horse serum. This is shown in tubes 6 and 7. Fixation in tube 8 and partial fixation in tube 8a, seems to us to indicate that a certain amount of antibody is dissociated to the salt solution from the sensitized pneumococci and that this supernatant fluid may contain a certain amount of dissolved antigen-antibody complex is indicated in tube 10; but the amount is reduced, as in tube 10a this irregularity disappears.

*Experiment III*

Pneumococcus Type I, grown on blood agar in large pie plates. Washed off in salt solution and thrown down in centrifuge. Washed twice in salt solution. Sediment shaken up in salt solution and 9 cc. of suspension sensitized with 0.9 cc. of antipneumococcus rabbit serum 446. Placed in water bath for one hour until firmly agglutinated, then in ice chest over night. Next morning centrifuged and, when densely packed, supernatant fluid is pipetted away and sediment rapidly washed over with salt solution without emulsifying, just to remove traces of serum-dilution not removable with pipette.

Sediment of sensitized pneumococci was then taken up in salt solution and divided into two portions "A" and "B." To "A" is added pneumococcus horse serum sufficient to make 0.1 cc. to every cubic centimeter volume. To "B" is added a similar amount of salt solution.

| | PNEUMOCOCCUS RESIDUE ANTIGEN | ANTIPNEUMOCOCCUS RABBIT SERUM 446 | | ANTIPNEUMOCOCCUS HORSE SERUM | | ALEXIN | | | RESULT IN TERMS OF FIXATION |
|---|---|---|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | | | | *units* | | | |
| 1 | 0.2 | 0.05 | Water bath 37.5°C. one-half hour | | Ten minutes at room temperature | 2 | Water bath 37.5°C. one hour | Sensitized red blood cells | ++++ |
| 2 | 0.2 | 0.05 | | 0.05 cc. unabsorbed | | 2 | | | 0 |
| 3 | 0.2 | 0.05 | | 1.0 cc. "A" | | 2 | | | ++++ |
| 4 | 0.2 | 0.05 | | 0.5 cc. "A" | | 2 | | | ++++ |
| 5 | 0.2 | 0.05 | | 0.1 cc. unabsorbed | | 2 | | | 0 |
| 6 | 0.2 | | | 1.0 cc. "A" | | 2 | | | ++++ |
| 7 | 0.2 | | | 0.05 cc. "A" | | 2 | | | ++++ |
| 8 | 0.2 | | | 1.0 cc. "B" | | 2 | | | ++++ |
| 8a | 0.2 | | | 0.5 cc. "B" | | 2 | | | + to++ |
| 9 | | | | 1.0 cc. "A" | | 2 | | | ++++ |
| 9a | | | | 0.5 cc. "A" | | 2 | | | ++++ |
| 10 | | | | 1 cc. "B" | | 2 | | | ++++ |
| 10a | | | | 0.5 cc. "B" | | 2 | | | 0 |
| 11 | 0.4 | | | | | 2 | | | 0 |
| 12 | | | | | | 2 | | | 0 |

Although we could absorb out the interfering body from the horse serum, however, we could not conclude from this experiment that the residue of the horse serum represented in supernatant fluid "A" now contained active alexin fixing sensitizer. Although such supernatant fluid (in tubes 6 and 7) gave definite fixation, nevertheless, when tested in similar amounts, but without antigen (in tubes 9 and 9a) fixation still occurred. In trying to overcome this difficulty, it was noticed that when the horse serum was added to the sensitized pneumococci, shaken up with them in salt solution and allowed to stand, no amount of subse-

quent centrifugation would completely remove a certain amount of turbidity. This seemed to mean that when the sensitized pneumococci were emulsified in salt solution, a certain amount of antigen became dissolved, that this reacted with the horse serum in a precipitate so fine that centrifugation could not remove it. This would naturally explain the variation appearing in tubes such as those indicated as "9" and "9a" in which this distinctly turbid supernatant fluid was mixed with alexin and subsequently tested for fixation. In order to overcome this we determined to filter the supernatant fluids "A" which represented the supernatant fluid left after horse serum had been absorbed with sensitized pneumococci in a salt solution suspension. The following Experiment IV, therefore, represents a repetition of Experiment III with the sole difference that both the unabsorbed horse serum and the supernatant fluid from mixtures of horse serum with sensitized pneumococci (namely, absorbed horse serum "A") were filtered through Berkefeld filters and reduced in quantities to such a degree that small remaining amounts of fixing complexes might be diluted to extinction.

*Experiment IV*

| | PNEUMOCOCCUS RESIDUE | | ALEXIN | 15° | 30° |
|---|---|---|---|---|---|
| | *cc.* | | *units* | | |
| 1 | | | 2 | 0 | 0 |
| 2 | 0.2 | 0.05 cc. rabbit serum 446 | 2 | ++++ | ++++ |
| 3 | 0.2 | 0.5 cc. (1:10 horse serum) | 2 | 0 | 0 |
| 4 | 0.2 | 0.5 cc. (1:10 horse serum) filtered | 2 | 0 | 0 |
| 5 | 0.2 | 0.5 cc. A | 2 | ++++ | ++++ |
| 6 | 0.2 | 0.25 cc. A | 2 | ++++ | ++++ |
| 7 | 0.2 | 0.5 cc. A filtered | 2 | ++++ | ++++ |
| 8 | 0.2 | 0.25 cc. A filtered | 2 | ++++ | ++++ |
| 9 | | 0.5 cc. A | 2 | ++++ | ++++ |
| 10 | | 0.25 cc. A | 2 | ++++ | ++++ |
| 11 | | 0.5 cc. A filtered | 2 | ++++ | ++++ |
| 12 | | 0.25 cc. A filtered | 2 | 0 | 0 |

To analyze this experiment, then, tubes 3 and 4 show that pneumococcus horse serum, both filtered and unfiltered, in

quantities of 0.5 cc. of 1 to 10 dilutions give no fixation of alexin with the "residue" antigen. Tubes 5, 6, 7 and 8, show fixation when the pneumococcus residue is sensitized with horse serum absorbed by sensitized pneumococci and placed in contact with the residue antigen both in the filtered and unfiltered condition. Tube 12 indicates that when quantities as low as 0.25 cc. of the filtered supernatant fluid "A" are used, fixation occurs in the presence of residue antigen, but no fixation when this supernatant fluid is used alone.

This result has been repeated with similar effect, and Experiment IV can be interpreted as indicating that alexin fixing sensitizer may be found active in horse serum from which the interfering body has been absorbed with sensitized pneumococci. On the other hand, we do not regard this experiment as conclusive, and it is still possible that the interfering substance, whatever it may be, is in actual combination with the sensitizer and that when it is absorbed out most of the sensitizer goes with it. Indeed, what we have designated as an interfering body may be nothing more than a peculiar state of the sensitizer which in some way changes the sensitized antigen so that alexin will not be fixed. On this basis, however, it would be difficult to explain why the horse serum should prevent the fixation of alexin by pneumococcus antigen sensitized with antipneumococcus rabbit serum. A final decision on these problems must be left to further work which is being continued on a more comprehensive scale in the laboratory.

## SUMMARY

The essential points presented in this paper are:

1. Certain antipneumococcus horse sera, though showing powerful precipitation reactions with pneumococcus antigen, do not sensitize similar antigens to alexin fixation.

2. Such sera not only fail to fix alexin in the presence of antigen as described above, but, in addition to this, they will prevent alexin fixation when added, in small amounts, to tubes of pneumococcus antigen sensitized with antipneumococcus rabbit serum. They, thus, prevent the fixation of alexin by a sensi-

tized antigen which would ordinarily have given a positive fixation. In order to obtain this interfering action, it is necessary to add the horse serum to the sensitized antigen before the alexin is added. If the alexin is added first and given thirty minutes at 37.5°C., subsequent addition of the horse serum does not interfere with the fixation.

3. This interfering effect of the horse serum can be removed by absorbing with sensitized pneumococci.

4. While such absorption completely removes the interfering effect from the horse serum, our experiments attempting to show that a fixing sensitizer is then left unhampered in the horse serum have not been conclusive up to the present time, but experiments like Experiment IV of the reported series suggest such a condition. This is being subjected to further study.

We cannot positively determine, as yet, whether interference with the attachment of alexin is due to a hitherto undescribed substance in horse serum, separate from the sensitizer, or whether the phenomenon depends upon adventitious conditions, such as the association of the sensitizer with serum constituents which alter its colloidal state or in some other way indirectly modify the physical conditions usually existing in such immune sera.

However this may be, our results appear to indicate definitely that the failure of a specific immune serum to sensitize antigen for alexin fixation, while powerful in its agglutinating and precipitating effects, does not prove the absence of a "fixing" antibody. This confirms our view that the failure of any individual antibody effect in an immune serum may be due to secondary and environmental causes rather than to absence of a given "antibody."

Incidentally, the experiments show that the titration of the antibody potency of horse serum for therapeutic use is not a reliable method, since such sera, extremely powerful in their agglutinating and precipitating effects and containing a high concentration of antibodies, may be entirely incapable of giving rise to alexin fixation reactions for the reasons demonstrated.

Whether or not the property observed by us is universal for horse sera or is at all common for immune sera in general has

not been determined as yet. It may well be that the body we have described is a constituent of exceptional specimens only. Because of its possible practical importance, however, this matter will be extensively investigated both in connection with pneumococcus and other sera.

In closing, we would again call attention to the fact that the residue antigens produced as described, both with pneumococci and a great many other bacteria, constitute the most easily managed and convenient antigens for specific precipitations and complement fixations. With the pneumococcus, it is merely necessary to grow on agar surfaces properly enriched, wash off with salt solution, shake for an hour in slightly alkalin salt solution, filter, boil with acid, clear by filtration and neutralize. The resultant water-clear fluid precipitates powerfully and possesses none of the disturbing features for complement fixations possessed by bacterial suspensions or extracts.

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# ON THE CLASSIFICATION OF THE PHENOMENA OF HYPERSENSITIVENESS[1]

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## I

BY ARTHUR F. COCA

Robert Doerr was the first to recognize the need of a classification of the phenomena of hypersensitiveness. In attempting such a classification Doerr (7), unfortunately chose as the main heading the term Allergie (allergy) which had been introduced by von Pirquet. This choice was particularly unhappy because Doerr felt obliged in using it to observe with some strictness the etymological significance of the term (altered reactivity). The result of this observance was the inclusion in Doerr's category of allergies of phenomena of such different nature as to make their association valueless if not positively confusing. There seems, for example, to be no advantage in associating in one category two so widely different phenomena as the well known morphine tolerance and morphine idiosyncrasy.

Doerr's subdivision of "allergic" phenomena into those exhibited to non-antigenic substances and those exhibited to antigenic substances is also inadvisable because such subdivision necessitates a confusing separation of identical conditions of hypersensitiveness in human beings, merely in accordance with the presence or absence of antigenic property in the exciting agent. Actually it seems that neither of Doerr's published

[1] Read at the annual meeting of the American Association of Immunologists, Washington, D. C., May 1, 1922.

classification recognizes the existence of the idiosyncrasies to antigenic and non-antigenic *proteins*.

In a summary of the literature of hypersensitiveness (3) the writer expressed the above outlined criticism of Doerr's classification and suggested as a main heading the word hypersensitiveness. This word was already in general use in the literature of anaphylaxis; the writer merely proposed that it be recognized in immunological parlance as a technical term ("terminus technicus," Doerr (8)) to signify any form of specific peculiar reactivity in which the characteristic symptoms were different in the different animal species and were different from the normal physiological reaction to the respective agent.

Such technical use of a common word has many precedents which show that no confusion need result from the practice. The restricted use in medical literature of the common word plague, for example, has resulted in no misunderstanding.

The writer's definition of hypersensitiveness in the immunological sense was drawn from the cardinal facts of anaphylaxis and of the human conditions of hay-fever, asthma, and other idiosyncrasies; it excluded, therefore, the sensitiveness of tuberculous individuals to tuberculin. The separation of tuberculin sensitiveness from the other two forms is amply warranted by the clearly marked differences among the three phenomena; however, it would be a mistake, as Doerr (8) points out, not to recognize tuberculin reactivity as a form of immunological hypersensitiveness and this the writer now acknowledges.

As Doerr remarks, tuberculin hypersensitiveness is the best-studied representative of a group of conditions of hypersensitiveness to derivatives of microörganisms. The list of these microörganisms is ever increasing and already includes *Sporotrichum*, Timothy Bacillus, *Trichophyton*, *B. mallei*, *B. abortus bovinus*, *B. melitensis*, *Spirochaeta pallida*, and *B. typhosus*, in addition to *B. tuberculosis*.

Since the known facts concerning this group of hypersensitive conditions indicate an identity in the underlying mechanism of all of them, an inclusive term seems to be needed to designate the group. The designation "cutaneous hypersensitiveness,"

which was employed by Fleischner and Meyer, seems quite inadequate because on the one hand the sensitiveness in infections is by no means confined to the skin and on the other hand cutaneous sensitiveness is by no means confined to infections. The designation "bacterial hypersensitiveness" seems objectionable because infection with fungi causes the form of sensitiveness that we are considering and because anaphylactic sensitiveness can be produced with bacteria.

It is proposed, therefore, to use for the present the unwieldy expression *hypersensitiveness of infection*, which could still be used even if it should be found that this form of hypersensitiveness can be produced without actual infection with the respective microörganisms.

Hypersensitiveness in its immunological sense can be defined as a susceptibility in man and animal that is mediated by a special mechanism, which may be specifically influenced (toward increased or diminished sensitiveness) by the suitable administration of the exciting agent.[2] This definition permits the inclusion under one heading of all of the related forms of hypersensitiveness. In most of these the sensitiveness is diminished by the administration of the exciting agent (hay-fever, asthma, etc., dermatitis venenata, anaphylactic sensitiveness); in ordinary serum disease and in drug idiosyncracy the reaction is often increased at the second administration. The existence of the special mechanism is indicated either by the absence of the sensitiveness in most individuals of the same species or by its total absence in other species. For example, only previously treated animals in whose tissues precipitating antibodies are present exhibit the usual form of anaphylactic sensitiveness. The "reversed" form of anaphylactic sensitiveness presents an exception to the rule in that it appears that one of the essential factors of the mechanism (the antigen), which is responsible for this form of sensitiveness, is present in the tissues of all individuals of the species. In this case, however, the existence of the mechanism is betrayed by the specificity of the injected immune serum.

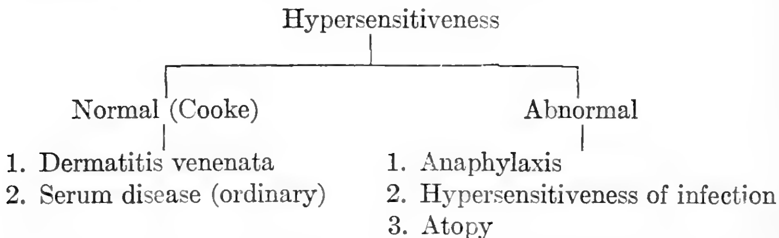
[2] This definition was formulated in conference with Dr. Cooke.

When the exciting agent possesses a normal physiological action the mechanism of hypersensitiveness is revealed in the well known qualitative difference between the symptoms of hypersensitiveness to the substance and those of the normal physiological effect.

The existence of the mechanism in *dermatitis venenata* can be inferred from the exclusive susceptibility of human beings. In the hypersensitiveness of infection the existence of the mechanism is evidenced by the lacking sensitiveness of normal individuals to the active agents; for example, tuberculin.

For reasons that are discussed in the second part of this communication, Dr. Cooke suggests the subdivision of the forms of hypersensitiveness into a "normal" group and an "abnormal" group. The group of abnormal hypersensitiveness includes anaphylaxis, the hypersensitiveness of infection and those idiosyncrasies that are controlled by the dominant gen demonstrated by Cooke and Vander Veer. This latter sub-group evidently needs a special term by which it may be conveniently designated and this need is satisfactorily met with the word atopy, which was kindly suggested by Professor Edward D. Perry of Columbia University. The Greek word ατοπία, from which the term was derived, was used in the sense of a strange disease. However, it is not, on that account, necessary to include under the term all strange diseases; the use of the term can be restricted to the hay-fever and asthma group.

The classification here proposed is thus:




The three abnormal forms are distinguished by the following features:

*Anaphylactic sensitiveness*

1. Is exhibited only against precipitinogenic proteins (Doerr).
2. Is passively transferable to normal animals with the serum of sensitized animals.
3. Is completely removable by the procedure of desensitization.
4. Is not inherited.
5. Is expressed, in the acute reaction, in symptoms and pathological changes which differ *characteristically* in the guinea-pig, the rabbit and the dog.

*The hypersensitiveness of infection*

1. Is exhibited against non-precipitinogenic substances; for example, tuberculin, which seems to be, as Doerr suggests, secretory products of bacterial growth.
2. Is not passively transferable to normal animals with the serum of sensitive animals.
3. Seems to be greatly lessened if not completely removable by the suitable injection of the active substance.
4. Is not inherited.
5. Is expressed, in the acute general reaction as well as in the cutaneous reaction, in symptoms and pathological changes which do not differ characteristically in the different animal species but which do differ from the characteristic symptoms and pathology of acute anaphylactic shock in the respective animal.
6. Is not developed after injections of the active substance, but regularly develops in the course of an infection with the microörganisms. The injection of the killed cultures results in anaphylactic sensitization to the injected material.
7. Seems in tuberculosis to be passively transferable to normal guinea-pigs by the injection of the crushed tubercles from infected guinea-pigs (Bail, 1, 2).

*Atopic hypersensitiveness*

1. Is exhibited against non-precipitinogenic substances as well as against precipitinogenic substances.
2. Has not been shown to be passively transferable to normal individuals with the blood of sensitive individuals. The one observation of Ramirez needs confirmation, being susceptible of a different explanation.

3. Can be greatly lessened but not completely removed by the suitable injection of the active substance (Cooke, 5).

4. Is inherited, subject to a dominant gen (Cooke and Vander Veer, 6).

5. Is expressed in pathological changes the more important of which are different from those of the anaphylactic reaction in the guinea-pig, the rabbit or the dog.

The writer's review of the subject of hypersensitiveness was written from the point of view of a proposed new classification and the description of the symptoms and pathology of the different forms of hypersensitiveness was devoted almost exclusively to the characteristic phenomena in each instance. Thus, the bronchiospasm in the guinea-pig, the arteriospasm in the rabbit, the hepatic congestion in the dog and the symptomatic effects of these changes were placed in the foreground with the purpose of emphasizing the wide difference among them as one of the cardinal criteria of anaphylactic hypersensitiveness. This criterion was added to the several others on which the separation of tuberculin sensitiveness from anaphylaxis was based.

Doerr objects to the prominence given to this difference on the ground that it is merely the necessary consequence of the special organization of each animal species and he points to the different physiological reaction of the dog, the cat, and the rabbit to morphine as an analogous situation. Doerr's objection here is, however, beside the point, as an examination of his cited analogy will show. If the typical action of an unknown alkaloid does not differ in the three animals that Doerr mentions in the characteristic manner of morphine, then the unknown alkaloid cannot be morphine. Similarly, if any phenomenon of hypersensitiveness fails in its typical expression to exhibit in the guinea-pig, the rabbit and the dog the differences which characterize the phenomenon of acute anaphylactic shock in these three species, then the phenomenon of hypersensitiveness in question cannot be that of anaphylaxis.

In continuation of his objection to this point of differentiation Doerr attempts to establish an identity of the characteristic symptoms of atopic hypersensitiveness and some of the symptoms of anaphylaxis.

Doerr likens the flow of tears, which sometimes occurs in anaphylactic shock to the condition of hay-fever in which also there is a flow of tears. However, lachrymation alone does not constitute hay-fever and there is no other ground for identifying this occasional minor symptom of anaphylaxis with the condition of atopic coryza. In other words, there is no evidence that the occasional flow of tears in anaphylactic shock is caused by a primary irritation of the conjunctiva.

To Doerr gastro-intestinal symptoms are of identical significance whether, as in the anaphylactic shock of the dog, they are due to passive congestion of the portal tributaries, or to a direct irritation of the gastro-intestinal mucous membrane as in the hypersensitive human being.

The asthmatic dyspnoea, which Doerr likens to the bronchiospasmic asphyxia of anaphylaxis in the guinea-pig is more than probably not due to contraction of the bronchial muscles, but to an edematous swelling of the bronchial mucous membrane.

Finally, Doerr thinks that the cutaneous manifestations of human hypersensitiveness (eruptions) may be represented among the anaphylactic symptoms of the guinea-pig by an itching of the skin which he assumes to exist on account of the fact that this animal often scratches its nose, ears and paws in protracted anaphylactic shock. However, there is no way of finding out whether the cause of the scratching is really situated in the skin or elsewhere, perhaps in the lungs. Those who use ether anesthesia in guinea pigs will recall the efforts to scratch the upper parts of the body which these animals regularly make after the ether has been administered for a time. One is not obliged to look upon these efforts as indicating an effect of the ether upon the skin; they may be due to an irritation of the lungs.

The grounds for separating the three forms of abnormal hypersensitiveness are sufficiently plain in the outlines of their prominent features given above. The grounds for their association must also be borne in mind; they have been set forth in the definition of hypersensitiveness. Dr. W. J. Elser[3] has sug-

[3] Personal communication.

gested that the special mechanism may be actually nothing positive, but only the absence of some normal element. This suggestion, however, is confronted with the difficulty, under such a hypothesis, of explaining the specificity that has been so constantly exhibited on the hyposensitization of atopic conditions (Cooke and Vander Veer). The hypothesis would need the supporting assumption that a different normal element is lacking in the hypersensitiveness to each exciting agent.

Admitting, then, the greater likelihood that the mechanism of the different forms of hypersensitiveness is something positive, one is naturally influenced by the example of anaphylaxis to imagine that the mechanism of the other two forms is essentially of antibody-antigen nature.

Doerr actually assumes the existence of anti-tuberculin antibodies in explanation of the tuberculin reaction and one is, indeed, tempted to follow him here; because one may well ask why a specific, positive mechanism that is developed during infection and that reacts specifically with a product of the respective microörganism may not be called an antibody.

The remarkable differences between the conditions under which this "antibody" is developed and reacts and those controlling the development and reaction of the known antibodies demand explanation.

One of the outstanding difficulties confronting the antibody-antigen conception of the tuberculin hypersensitiveness is the passive transferrence of the sensitiveness to the normal animal by the injection of crushed tubercle tissue. This procedure makes it necessary to assume that the anti-tuberculin "antibodies" are present in the tubercle tissue. Since tuberculin sensitiveness does not develop without the formation of tuberculous tissue (Krause-Doerr) one must further conclude that the hypothetical antibodies are produced only by tuberculous tissue. Doerr goes so far as to say that only tuberculous tissue reacts with tuberculin and he offers this as the explanation of the characteristic difference between the tuberculin reaction, and that of anaphylaxis.

But Doerr seems to have overlooked here the reactivity of the

skin, conjunctiva and uterine muscle (all non-tuberculous tissues) in tuberculosis. The mystery of the anti-tuberculin antibodies is deepened by the experiments of Fellner, which confirm, in a way, those of Bail in the passive transferrence of tuberculin sensitiveness. Fellner obtained the reaction tissue resulting from a von Pirquet cutaneous test and inoculated it, mixed with tuberculin, into the skin of a normal; that is, tuberculin-negative individual. This inoculation resulted in a distinct reaction which was absent or very weak in the control inoculation with the tissue alone. It would seem from this experiment that newly formed tubercle tissue even when it is far removed from the focus of infection; that is, from the tubercle bacilli, quickly begins to produce anti-tuberculin antibodies.

The anti-tuberculin antibody is not produced in normal animals by the injection of tuberculin, with which it reacts. It is produced only in tubercle tissue, but it is diffused out of this parent tissue into the other tissues of the body where, also, it is capable of reacting with tuberculin. Notwithstanding this demonstrated diffusibility, the antibody has not been demonstrated in the blood. With one exception, the uterine muscle, the tissues thus, so to speak, passively autosensitized are not those that are involved in anaphylactic sensitization. The skin is not sensitive in anaphylaxis and the bronchial muscles are not sensitive in the tuberculous guinea pigs.

Finally, passive sensitization in anaphylaxis has not been accomplished by the transfer of any tissue of a sensitive animal excepting the blood. The exact reverse of this is true in tuberculin sensitiveness.

The foregoing parallel summary of the facts concerning the anti-tuberculin antibody and the anaphylactic antibody reveals an antithesis that would seem to discourage the attempt to ascribe an antibody-antigen nature to the tuberculin reaction. Nevertheless, this hypothesis appears to be the most promising at present, although further, convincing confirmation of the experimental basis of the hypothesis is demanded.

For an assumption of an antibody-antigen nature of the mechanism of atopic hypersensitiveness there seems to be no reasonable evidence whatever.

In the writer's previously published discussions of this question this conclusion was drawn from the first four features of atopic hypersensitiveness mentioned above. These, more briefly stated, are:

1. The non-antigenic property of some atopens.
2. The absence of proof that atopic hypersensitiveness can be passively transferred.
3. The absence of desensitization in atopy.
4. The determining influence of the atopic dominant.

One of the results of Doerr's definition of the word "allergie" was the inclusion under that heading of both "toxin hypersensitiveness" and antitoxic immunity.

In the author's review in Tice's Practice of Medicine, it was pointed out that whenever the exciting agent of hypersensitiveness possessed a normal physiological action that action was nearly always different from its effect in the hypersensitive individual. This difference was proposed as a criterion by which the existence of the mechanism of hypersensitiveness can be determined and in accordance with that criterion the so-called toxin hypersensitiveness was excluded from the category of what was then referred to as true hypersensitiveness; because the symptoms of "hypersensitiveness" to the toxin were always those of the normal action of the toxin.

The later study of Park[4] has further justified the exclusion because Park finds that there is no hypersensitiveness to toxin; the phenomenon that was formerly considered toxin hypersensitiveness is merely a lessening of the primary lethal dose by means of fractional administration. According to Park, this phenomenon is observed only within the period previous to the production of antitoxin; that is, before the specific reaction mechanism has appeared.

Doerr rests his grouping of antitoxic immunity with the phenomena of anaphylaxis on the ground that "the toxins are antigens, which are neutralized by their antibodies exactly as are the anaphylactogens." But specific neutralization is not

[4] Read at the annual meeting of the American Association of Immunologists, Washington, D. C., May 1, 1922, unpublished.

the criterion by which the anaphylactic state is judged as is evident in the fact that such neutralization of precipitin with precipitinogen takes place in the blood of guinea-pigs without the development of anaphylactic shock; it is not the neutralization of the antibody in the animal body that counts, but the resulting *irritation* of the cells. Anaphylactic shock is not, as Doerr would define it, "an antibody-antigen reaction," but the secondary effect of such a reaction. Since neither toxin nor antitoxin enter into any phenomenon of hypersensitiveness it would be only confusing to consider their mutual relationship under that heading.

The question as to the possibility of the occurrence of anaphylactic sensitization in human beings remains undetermined.

Doerr has misunderstood the writer's attitude upon this question, remarking in one place that (according to Coca) "the human being cannot be sensitized, cannot be made anaphylactic." Actually the writer wrote in two places as follows:

> . . . . it seems necessary to conclude: first, that if anaphylaxis does occur in man, it does so only very rarely and secondly, that there is no positive evidence that anaphylaxis occurs at all in human beings (4).
>
> In conclusion it may be said that, while it is not possible to assert that anaphylaxis does not occur in human beings, it is a fact that the existence of the condition of anaphylaxis in human individuals has not been demonstrated. . . . .

We find ourselves, thus, in agreement with Doerr when he writes:

> *It is not, for example, impossible* that the sharp distinction drawn by Coca between idiosyncrasy (atopy) and anaphylaxis can be upheld, but *that both forms of hypersensitiveness appear in human beings*[5] (8).

In fact, the alteration of the writer's definition of hypersensitiveness to permit the inclusion of the hypersensitiveness of infection has removed Doerr's chief objection to the classification previously proposed and there is left no important disagreement with Doerr as to the essential nature of the associated phenomena.

[5] The italics are introduced by the writer.

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II

BY ROBERT A. COOKE

A classification of any branch of science usually carries with it an air of finality. The present classification is not suggested in that way for we recognize fully the many loopholes in our knowledge of the subject. On the other hand a classification undoubtedly aids in a proper alignment and grouping of the facts and serves, not as an end, but as a means of advancement in the study of a subject. The classification itself is not so important but the facts upon which it is based must be genuine, then, with further study and the correlation of new facts, a new grouping and classification may be needed. But it has seemed wise to attempt a classification of certain biological reactions, namely hypersensitiveness, in the light of present facts as we now see them.

Dr. Coca has just given a defence of the use of the word hypersensitiveness in a restricted immunological sense and it is a useful and satisfactory word to designate a certain group of biological reactions occurring in man or animal. In his article on Hypersensitiveness he says "If an individual reacts specifically or particularly with characteristic symptoms to the administration of or to contact with a quantity of any substance, which, to the majority of the members of the same species of animal is innocuous, that individual is said to be 'hypersensitive' to that substance." The term 'hypersensitiveness' must be relieved of some of the restrictions originally imposed by Coca and together we have formulated the definition which he has already given and which I repeat.

Hypersensitiveness is a state of susceptibility in man and animal in which the reaction is mediated by a special mechanism that occurs naturally or is developed artificially and which may be specifically influenced (increased or diminished) by the suitable administration of the exciting agent. In other words there are two criteria by which we distinguish hypersensitiveness from other biological reactions. First, it requires a special mechanism and secondly, it is susceptible to specific alteration.

The term "special mechanism," employed in the definition, unless explained, would connote a positive factor in the reaction such as antibodies in the mechanism of anaphylaxis. It must be understood however that we do not know the mechanism of the reaction in atopy. It is conceivable that it is due to the absence of some normal bodily constituent of an, at present, unknown nature. Certain it is that the reaction is not mediated by any demonstrable, positive factor, such as the anaphylaxis antibody. The term special mechanism then is used to denote a procedure which may depend upon either a positive or a negative factor.

Since the nature of the reaction is unknown in certain forms of hypersensitiveness two criteria have been formulated by which to determine the presence of a special mechanism when the establishment of the mechanism is not controllable and artificially induced as it is in anaphylaxis and infection. First, the absence of the reaction in a considerable percentage of the same species as, for example, atopy occurring in only ten per cent of the white race. Second, the absence of the reaction in other mammalian species though present in one, as serum disease occurring in ninety per cent of the Caucasian race but absent in other mammalia.

With these points clearly in mind we can exclude reactions to chemical, thermal and mechanical irritants and also the ordinary pharmacological reactions of drugs, but not some of the so-called drug idiosyncrasies, which are genuine forms of atopy, some inherited, some probably induced.

The normal or pharmacological action of drugs depends for the most part upon a direct action of the drug upon certain tissues or cells and this is essentially identical for all mammalia

and even lower animals. In other words, in pharmacology the action of drugs is qualitatively similar throughout the mammalian group, although effects may be apparently widely different, but the reasons for the differences appear on critical analysis. For example atropin has the same action, paralyzing the vagus, in man and in rabbits, but the effects are widely different in that vagus paralysis in man commonly causes marked cardiac acceleration but, in the rabbit, in the absence of tonic vagus impulses, this does not occur. Again, morphine in the frog induces strychnine-like convulsions whereas these are not apparent in man. But the pharmacologists know and have very good proof of the fact that morphine does markedly increase the reflex irritability of the anterior horn cells in man and convulsions are only prevented because death results early from the depressant effect of the drug on the respiratory centre. Paralysis of the respiratory centre does not immediately cause death in the frog.

Another type of differences in drug effects is seen in the apparent tolerance of the frog to colchicin. This drug is highly toxic in all warm blooded animals. The tolerance of the frog entirely disappears when its temperature is elevated to that of the warm blooded animals. Colchicin is not decomposed or oxidized to its toxic constituents except at a temperature higher than that which is normal for the frog.

Many other examples might be cited but these suffice to show that the normal action of drugs is not mediated by any special mechanism, that the action is qualitatively similar throughout the mammalian group. To be sure, there are certain variations in the effect of drugs within the same species. Enormous individual variations occur to most every drug such as caffein, strychnin, digitalis and histamine, but these are purely quantitative.

To certain drugs, notably morphine, alcohol and nicotine, there is an acquired tolerance. Let us examine the facts in regard to the drug tolerance to see whether or not it bears any relation to the specifically altered reactions of hypersensitiveness. In other words is drug tolerance a form of immunity, desensitization, or hyposensitiveness?

The tolerance to alcohol induced by habitual use is non-specific. An increased tolerance to ether and chloroform is created at the same time and, further, the increase of this tolerance is very limited and very gradually developed. What about morphine tolerance! In the first place the tolerance to morphine is very limited, it is never increased to more than twenty times the fatal dose and usually only four to five times and this only after long continued use. If a man is tolerant to four times the fatal dose, five times the fatal dose will kill with the same symptoms and in the same time that the drug acts upon a normal man. Again the tolerance acquired to morphine is limited to the cells of the cortex and respiratory centre. The other morphine effects in the body, cardiac, intestinal, etc., proceed without alteration.

The pharmacologists do not conceive drug tolerance as in any way comparable to immunity, desensitization, or hyposensitization. Without further discussion then it would seem that the word hypersensitiveness in its immunological sense and as defined is free of the danger of being confounded with any biological reactions occurring in the realm of pharmacology.

Under the general heading Hypersensitiveness we at once recognize the three distinct types of reactions which were originally distinguished by Coca, namely anaphylaxis, atopy, and the sensitiveness of infection. They are forms of hypersensitiveness because they depend each upon its own special mechanism for the production of symptoms. What this mechanism may be in atopy we do not know today, but, whatever it is, it develops naturally in man as a result of certain inherited factors. There is one feature that is common to these three states and that is a qualitative cellular alteration within the species. The anaphylactic or sensitized guinea-pig is qualitatively different from the normal pig. The atopic man is qualitatively unlike the normal man. To be sure there are variations in degree of sensitiveness within each group. Guinea-pigs may be relatively more or less anaphylactic and individuals may be relatively more or less atopic. But these are purely quantitative variations and independent of the underlying fundamental qualitative change.

For this reason we have grouped these three states under a subheading, abnormal—abnormal hypersensitiveness dependent upon qualitative differences within the species. But this connotes the necessity for a subheading of normal—normal hypersensitiveness dependent only upon quantitative variation within the species and such forms I believe we have in two clinical conditions of man; namely, serum disease and dermatitis venenata.

## SERUM DISEASE

In the first place we must not confound serum disease with the immediate reaction occurring in human atopy. They differ, first, in their pathogenesis, the one inherited and the other not; secondly, in their incubation period, the one instantaneous, the other after an interval of about five to fifteen days; thirdly, in their symptomatology, dyspnea from bronchial edema being the outstanding feature in atopy and urticaria the principal symptom in serum disease.

Nor must serum disease be confounded with the possible serum anaphylaxis occurring in man. I say possible, because in the nature of things it is not feasible to experiment on man, but a sufficient number of clinical cases is reported to make it seem probable that anaphylaxis does occur in man. But these clinical experiences are not yet supported by any absolute proof. By serum disease is meant those manifestations of urticaria, pruritus, joint pains, and febrile movement, occurring after an incubation period of usually five to fifteen days.

From 1905 when von Pirquet and Schick announced their theory, the antigen-antibody nature of the reaction known as serum disease was accepted until 1920 when Coca's critical analysis of the facts threw grave doubts upon the correctness of this assumption. Hamburger and Moro, von Pirquet and Schick, C, W. Wells, Francioni, Longcope and Rackemann and Longcope and Mackenzie, have all studied serum disease from the point of view of von Pirquet's theory and the results of their studies fail to show any constant relationship between the symptoms of the disease and the specific precipitins in the blood and between the symptoms of the disease and the presence or absence

of antigen in the blood. If for no other reason, this lack of relationship alone is sufficient to overthrow von Pirquet's theory. If this contention is valid, as it appears, then serum disease is not anaphylaxis.

Heredity plays no part in serum disease. A few years ago, when serum was given as diphtheria antitoxin in relatively small amounts, the general incidence of serum disease was given as ten per cent. In 1916 the writer, with Vander Veer, had concluded from a statistical study that the general incidence of what we now call atopy was about ten per cent. The close approximation of the incidence percentage for serum disease and the natural human form of hypersensitiveness was the determining factor by which Coca then grouped these conditions as similar, under the heading of allergy.

With the introduction of an antipneumococcus serum, used in large amounts intravenously, the general incidence of serum disease has risen to ninety percent, according to the observations of Cole, Longcope and Mackenzie, as well as the statistics collected by Coca from the Boston City Hospital.

Another point recently brought forward by Coca is that the percentage incidence for serum disease is practically the same, ten to thirteen per cent after subcutaneous injection, in all age groups, thus differing entirely from the percentage incidence of the inherited forms as shown by the figures obtained in 1916 by the writer with Vander Veer and later supported by Adkinson and again today by Spain. Nor, as mentioned above, is there any identity in the incubation period or the clinical symptoms. As a matter of fact the two reactions serum disease and serum atopy have nothing in common other than a common inciting agent. The immediate reaction of atopy and the delayed reaction of serum disease may and may not appear in the same individual as a result of the same dose of serum. Clinically we see violent immediate reactions with, later, marked serum disease, mild serum serum disease or no serum disease at all and, on the contrary, we see marked serum disease with no immediate reaction or a mild immediate reaction may have occurred. That they do co-exist is due solely to the fact that serum disease occurs

in 90 per cent of all humans. This quantitative symptomatic independence of the two reactions in individuals susceptible to both certainly connotes an independent mechanism for each reaction. It would then seem logical to conclude that serum disease is not a manifestation of atopic hypersensitiveness.

The question may then well be asked, is serum disease a form of hypersensitiveness; that is, is it dependent upon a special mechanism and is the reaction specifically influenced as the result of the administration of the exciting agent?

Serum disease as seen in man cannot be reproduced in animals[6] and further, as recently shown by Coca in his study of serum disease in the American Indian, this race is much less susceptible to the disease, only 46 per cent, than is the white man in which over 90 per cent are susceptible.

With regard to the second point—alteration of the reaction by administration of the exciting agent—the observations of von Pirquet and Schick supported by Goodall and by Currie, have established the fact that there is a definite shortening of the incubation period following reinjection and, though not the rule, von Pirquet and Schick and Goodall have reported cases with an entire absence of symptoms of serum disease on reinjection when the first injection had given typical manifestations. But it must be borne in mind that these alterations of reaction do not conform to the principles of desensitization or anti-anaphylaxis nor to the phenomenon of hyposensitization of atopy.

### DERMATITIS VENENATA

For some years we have undertaken, more or less casually, a study of the irritants in poison ivy and sumac. These are readily extracted in 95 per cent alcohol and in chloroform, as shown by the fact that a small amount of the extract applied to the intact skin produces the typical vesicular lesion. Dr. Spain recently undertook a more intensive study, and in a recently published work from our clinic, he has shown that the eruption occurs only

[6] This is true with the single exception reported by Zinsser of what appeared to be serum disease in one monkey.

after an incubation period of from two to fourteen days, in one case twenty-four days. He further showed that the typical vescular lesion could be produced by test in 64 per cent of 104 cases, including children and adults. More recent and still unpublished work indicates that this percentage may be considerably increased for in no case tested with the stronger sumac extract has the reaction failed, except that no reaction has yet been obtained with the strongest extracts in infants before the age of three.

Other facts elicited are that this extract is non-toxic for animals when injected intravensouly and the skin of animals, dogs, rabbits and guinea pigs, is not in any way affected by the application of the extract to the skin. In other words the active principle is not a simple escharotic.

It is not necessary to argue that this reaction is not an anaphylactic reaction for it is non-antigenic. The reaction in man is not a form of atopy on account of the high general incidence and the fact that incidence by age groups does not show the influence of bilateral, unilateral and negative inheritance. That it is a form of hypersensitiveness however is evidenced by the fact that the reaction is mediated by a special mechanism that is absent in animals and is not developed in the human until the age of three. That the reaction can be specifically influenced by administration of the extract is evidenced in some still unpublished work but will not be further discussed here.

These two reactions observed in man, serum disease and dermatitis venenata, for the reasons given above would seem properly to be included as forms of hypersensitiveness and because of their incidence in such a large percentage of the white race are satisfactorily grouped under the subdivision of "normal" forms, showing only quantitative variation within the species.

### CONCLUSIONS

The term hypersensitiveness has been defined.

Biological or cellular activities in response to mechanical and thermal and chemical irritants, as well as the ordinary pharma-

cological reactions of drugs, are excluded as requiring no special mechanism and not subject to specific alteration by the use of the specific agent.

Hypersensitiveness is subdivided into:

1. Normal forms dependent only upon quantitative variations within the species, serum disease and dermatitis venenata have been discussed as examples.

2. Abnormal forms dependent entirely upon qualitative differences within the species. In this group belong: (*a*) anaphylaxis, an antigen antibody reaction: (*b*) atopy, the inherited human hypersensitiveness: (*c*) the sensitiveness of infection.

# STUDIES ON THE TOXICITY OF HUMAN BLOOD PLASMA FOR GUINEA-PIGS

## III. THE TOXICITY OF HUMAN BLOOD PLASMA IN THE VARIOUS STAGES OF PULMONARY TUBERCULOSIS

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Recently (1) we have reported the effect of intravenous injections of maternal and fetal plasma into guinea-pigs and observed that the shock effect was the result of an intravascular coagulation. In a subsequent study, it was observed that the toxic effect of the blood was greatest in those cases which contained a large amount of fibrinogen. For example, the maternal blood was more toxic to the guinea-pig than the fetal blood taken from the same case, by virtue of the fact that the maternal blood contains a larger amount of fibrinogen than the fetal blood.

As corroborative evidence of the above fact, it was also observed that any agent that will prevent clotting, such as hirudin when injected intravenously before the blood plasma was injected, or filtering the plasma through a Berkefeld filter will prevent an intravascular coagulation. These observations have also been noted in a somewhat similar manner by Sachs (2), Herzfeld and Klinger (3), and more recently by Starlinger (4). This latter investigator has noted that the flocculation of the blood plasma depends quantitatively upon the amount of fibrinogen present in the plasma, and that a partial removal of the fibrinogen by delicate absorption agents such as kaolin or bone black, diminishes or completely suppresses the degree of flocculation, and also the sedimentation of erythrocytes. These obser-

vations, along with those noted by Sachs and von Oettingen (5) have been made on bloods from pregnant and fetal cases.

Other conditions besides pregnancy may also show a quantitative increase in the fibrinogen content of the blood such as pneumonia, typhoid fever, and pulmonary tuberculosis. The latter disease being of a chronic type involves a destruction of tissue cells, followed by an attempt on the part of the body to repair the involved area. This destruction of tissue liberates a substance into the blood that may be one of the factors in stimulating the formation of fibrinogen, and we therefore undertook a study of the plasma of tuberculous patients to see whether or not the various stages of tuberculosis may show any differences as to the degree of the fibrinogen content of the plasma, and also if there might exist a difference as to the degree of toxicity in the various stages of tuberculosis.

Together with the subject of the toxicity of the blood plasma and the fibrinogen content in pulmonary tuberculosis the suspension stability of erythrocytes might also be taken into consideration. Fahraeus (6), Westergren (7), Frisch and Starlinger (8), and more recently Katz (9), have carried on a comprehensive research work upon the sinking velocity of erythrocytes in pulmonary tuberculosis. Westergren has shown that the sinking speed of the red cells is abnormally high in the majority of cases in which the pathologic process is active. Together with this observation and those made by other investigators (Abderhalden (10), Linzenmeyer (11), Büscher (12), and others) it has been shown that in those cases having a high degree of sedimentation of erythrocytes, the fibrinogen content of the blood plasma was correspondingly increased above normal.

Katz in determining the sedimentation speed of erythrocytes in the various stages of pulmonary tuberculosis has attempted to correlate the relationship of the suspension stability with the pulse-temperature curve. His results show that the speed of sedimentation is in proportion with the pulse and temperature rate, i.e., the higher the pulse and temperature the greater the speed of sedimentation. Starlinger has observed that in those cases where there is a great destruction of tissue cells, one observes

a marked sedimentation reaction, especially is this phenomenon pronounced in malignant tumors, pneumonia, florid lues, and pulmonary tuberculosis.

Because of the fact that the fibrinogen content seems to play such an important rôle in the two conditions; namely, the toxicity of the blood plasma and the suspension stability of erythrocytes, it was thought advisable to investigate the relationship these two conditions might have in tuberculosis. A more detailed study of the sedimentation reaction in pulmonary tuberculosis will be left for a subsequent study to be made along this line, and especial emphasis will be made as to its diagnostic and prognostic value.

## METHODS AND RESULTS

To determine the suspension stability of erythrocytes we have employed the technic of Fahraeus (6), using a test tube 150 mm. in length having an internal diameter of 9 mm. and graduated to 0.1 cc. 2 cc. of a 2 per cent sodium citrate is used, and the blood, drawn from the median basilic vein, is allowed to run into the tube up to the 10 cc. mark. The time is noted when the blood is collected. The tube is inverted 2 or 3 times to insure thorough mixing of the blood. At the same time blood is also collected for the toxicity test, and for a fibrinogen determination.

To determine the toxicity of the blood plasma taken in the various stages of pulmonary tuberculosis, we have used guinea-pigs which have not been previously sensitized to a foreign protein. The technic of injecting the plasma into the jugular vein has been previously described by us. For the determination of the fibrinogen content of the blood, Gram's (13) technic has been used.

In our previous studies on the toxicity of human blood plasma for guinea-pigs, we have shown that the toxic dose of normal male adult plasma was 1.5 cc. per 100 grams of guinea-pig weight, and for female adult, 0.75 to 1 cc. of plasma per 100 grams of guinea-pig weight. Table 1 shows that the average toxic dose of blood plasma in pulmonary tuberculosis is 0.5 cc. per 100 grams of guinea-pig weight; 0.25 cc. per 100 grams of guinea-pig weight

does not kill the animal. The toxic dose of blood plasma in pulmonary tuberculosis is the same as that of normal pregnancy and during delivery. It is also of interest to note that the toxic dose of blood plasma is practically the same in the various stages of tuberculosis.

It will also be observed (table 1) that the fibrinogen content of the blood plasma taken from the same cases is markedly increased, the rate of sedimentation corresponding to that reported by other investigators.

TABLE 1

*The toxicity of human blood plasma in the various stages of pulmonary tuberculosis, and its relation to the sedimentation of erythrocytes and the pulse-temperature range*

| CLASSIFICATION | TOXIC DOSE OF BLOOD PLASMA PER 100 GRAMS OF GUINEA-PIG WEIGHT | SEDIMENTATION OF ERYTHROCYTES* | | | FIBRINOGEN | TEMPERATURE RANGE | AVERAGE NORMAL PULSE |
|---|---|---|---|---|---|---|---|
| | | One hour | Two hours | Twenty-four hours | | | |
| | *cc.* | *cc.* | *cc.* | *cc.* | *grams* | *°F.* | *per minute* |
| Turban III... | 0.5 | 4.0 | 4.3 | 6.0 | 0.45 | 99.5 | 100 |
| Turban III... | 0.5 | 3.4 | 4.0 | 5.9 | 0.4 | 100.0 | 101 |
| Turban III... | 0.5 | 4.2 | 4.8 | 6.5 | 0.55 | 102.0 | 120 |
| Turban III... | 0.5 | 4.0 | 5.0 | 6.2 | 0.42 | 100.0 | 108 |
| Turban II.... | 0.5 | 3.0 | 4.4 | 5.0 | 0.4 | 99.4 | 100 |
| Turban II.... | 0.5 | 3.5 | 4.0 | 5.2 | 0.375 | 99.0 | 100 |
| Turban II.... | 0.5 | 4.0 | 4.3 | 6.0 | 0.382 | 99.6 | 102 |
| Turban II.... | 0.5 | 4.4 | 5.0 | 6.0 | 0.4 | 99.8 | 103 |
| Turban I..... | 0.5 | 3.0 | 4.0 | 4.8 | 0.375 | 99.0 | 96 |
| Turban I..... | 0.5 | 4.0 | 4.8 | 5.1 | 0.35 | 98.6 | 99 |
| Turban I..... | 0.6 | 3.6 | 4.5 | 5.0 | 0.39 | 98.8 | 100 |
| Turban I..... | 0.55 | 4.0 | 4.4 | 5.0 | 0.382 | 99.0 | 90 |

* Determined by measuring the height of the clear plasma layer above the lower column of red cells.

The relationship of the pulse and temperature ratio with the suspension stability is of interest, for it will be observed that a relationship exists between these factors. Although the pulse and temperature curve might not be employed to demonstrate the degree or extent of involvement in pulmonary tuberculosis it is, however, a factor in expressing activity and is an index in demonstrating tissue destruction. We have not collected blood several

times from each patient to show that the speed and degree of sedimentation of erythrocytes may vary with the pulse and temperature change. This correlation has been carefully studied by Katz.

## DISCUSSION

In view of the fact that tuberculosis as a rule follows a protracted course, one may presuppose that the continuous breaking down of tissue cells may liberate substances that induce the quantitative increase of fibrinogen in the blood. Just how long this active tissue destruction continues cannot always be determined. It is possible that the fibrinogen content may be of value in determining this clinically important question, although we have not at the moment had the opportunity of examining the plasma of recovered patients.

On previous observations with fetal and maternal bloods, the bloods of various diseases, and the results observed in the present study indicate that the degree of toxicity also depends upon the quantity of fibrinogen present in the blood.

In pulmonary tuberculosis there is an increased serum protein content which is especially evident in advanced phthisis, while the markedly cachectic cases reveal subnormal values. Frisch (14) has also shown that a markedly increased fibrinogen content exists in the nodose and pneumonic forms of pulmonary tuberculosis, the serous effusion and intestinal, the fibrotic and bronchial gland cases, while a rather small increase or normal finding was characteristic of the latent and dry pleurisy cases. Repeated examinations of the fibrinogen content showed characteristic variations going hand in hand with the clinical course. A parallelism exists between the presence of fever and the fibrinogen content of the blood. This observer is also of the opinion that the fibrinogen originates from the pathologic processes, which destroy the cell in the diseased areas and, therefore, can be taken as a measure of tissue destruction. Practically this test should differentiate active from inactive (latent) tuberculosis.

Becquerel and Rodier (15) have investigated the fibrinogen content of the blood in pulmonary tuberculosis and have observed

that the greater increase in the fibrinogen content usually occurs in those cases which show an increased activity. This observation is in accord with the findings of Foster and Whipple (16) who have shown that tissue injury and inflammation seem to increase the production of fibrinogen and cause a marked increase in the fibrin values. Mills (17) is of the opinion that the lungs yield the most potent product that is capable of activating the coagulation of the blood. This observer has shown that tissue fibrinogen has no toxic effect on the body, unless clotting of the blood is induced. Mills has also demonstrated that one must avoid injecting the substance directly into the circulating blood, as there is very great danger of immediate death from intravascular clotting, an observation similar to that recorded by us.

It is possible therefore, to associate the quantitative increase of fibrinogen in pulmonary tuberculosis with the degree of activity of the disease, as the fibrinogen factor may be used as an index of tissue destruction. Especially is this condition pronounced when it is observed that the degree of toxicity depends upon the quantitative increase in fibrinogen. Clinically, an increase in the pulse and temperature rate is only one of the methods by which cellular destruction may be expressed.

The question of sedimentation of erythrocytes was introduced into the present study to show the relationship this might have with the question of toxicity. Although a detailed discussion of the suspension stability must be left for another paper dealing with this subject, the factors influencing the speed of sedimentation might be enumerated. The consensus of opinion among the various investigators is that the sedimentation of the red blood cells depends upon the amount of fibrinogen present (Starlinger, Sachs, and others). Abderhalden (10) suggests the possibility that the sedimentation time might also depend, not only on the composition of the blood plasma, but also on that of the red blood cells themselves. Marloff (18) believes that the size and hemoglobin content of the erythrocytes have a certain effect on its sedimentation time. Plaut (19) is of the opinion that the rapidity of the sedimentation might be explained along the lines of auto-agglutination, which he thinks is dependent upon the

fibrogen content of the serum. Hans Sachs believes that the rapidity of sedimentation is due to a variation in stability of the plasma in which fibrinogen is the most labile component. But since the sedimentation of the red blood cells depends upon the amount of fibrinogen, and there exists a parallelism between sedimentation and toxicity, as we have brought out by our experiments, this alone is sufficient proof of the casual importance the amount of fibrinogen plays in the question of toxicity in pulmonary tuberculosis.

It is obvious then, that in those cases having an increased temperature and pulse rate, the toxicity of the blood plasma for the guinea-pig is increased; also an increase in the suspension stability of the red cells. As we have pointed out above, the toxicity of blood plasma depends upon the fibrinogen content of the latter, and this factor also plays an important rôle in the sedimentation of erythrocytes (Fahraeus, Gram). These four factors, temperature, pulse, toxicity, and sedimentation are parallel with each other. Although the degree of toxicity is no index as to the prognosis of the disease, and can not be used in diagnosis, it does show that the balance between pathologic activity (in tuberculosis) and the factors influencing a physiologic balance of the organism is disturbed.

## SUMMARY

As a result of tabulating the findings in the various stages of activity, it appears that the suspension stability of the blood in pulmonary tuberculosis parallels the activity of the process.

With an increase in the pulse rate and temperature, there is also an increase in the sedimentation rate of erythrocytes.

The toxicity of the blood plasma in pulmonary tuberculosis is also increased for the guinea-pig. The pulse, temperature, suspension stability of erythrocytes, and toxicity of the blood plasma are parallel with each other.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## IV. THE DOMINANT REACTING TISSUES IN PEPTONE SHOCK

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It is currently assumed that the acute anaphylactic shock and the peptone shock in dogs are physiologically identical reactions. This assumption is based on the marked similarity between the clinical pictures and pathological findings of the two reactions. It has been shown that the liver is the essential or dominant reacting organ in canine anaphylaxis (1). The assumption has, therefore, been made that canine peptone shock also is fundamentally an hepatic reaction. In order to test the validity of this assumption, we have studied the peptone reaction in dehepatized and eviscerated dogs.

### PEPTONE SHOCK IN DEHEPATIZED DOGS

The dehepatization tests were made with the simplified Eck-fistula technic recently proposed by Dale and Laidlaw (2). According to this technic connection is made between the portal vein and the inferior vena cava by means of an excised portion of the jugular vein of the same animal, Crile's transfusion cannulae being used to make the connections. In our initial tests we used the excised vena cava of a second dog for this connection. In our later tests we simplified the technic by dissecting out the abdominal vena cava of the animal to be tested and connecting its caudal end to the portal vein by means of a transfusion cannula. The portal vein and hepatic artery were then ligated immediately adjacent to the liver, and varying amounts of peptone injected intravenously. Typical data thus obtained are shown in table 1.

From this table it is seen that marked peptone reactions take place in dehepatized dogs. The liver therefore is not an essential or dominant organ in this reaction.

The reactions in dehepatized dogs, however, show constant quantitative differences from the control reactions in intact dogs. The fall in arterial blood pressure is never as pronounced as in the intact animal. From this we conclude that there is an important though not dominant hepatic factor in the production of this shock.

The most marked difference, however, is observed in the recovery period. In intact dogs the arterial blood pressure is usually restored to normal in from thirty to sixty minutes, depending upon the peptone dose injected. Practically no recovery takes place in dehepatized dogs. The liver therefore is undoubtedly the essential or dominant organ in the recovery from peptone shock.

TABLE 1

*Typical reaction in dehepatized dogs*

Intravenous injections of 0.25 gram Witte's peptone (B) per kilogram of body weight. Morphine-ether anesthesia

| ANIMAL TESTED | CAROTID BLOOD PRESSURE | | | | | | |
|---|---|---|---|---|---|---|---|
| | Initial | 90 seconds | 5 minutes | 10 minutes | 20 minutes | 40 minutes | 60 minutes |
| | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg |
| Dehepatized dog | 100 | 40 | 40 | 45 | 40 | 35 | 30 |
| Control dog | 110 | 30 | 22 | 28 | 40 | 65 | 90 |

## PEPTONE SHOCK IN EVISCERATED DOGS

To prepare dogs for the evisceration tests, the esophagus, descending colon, inferior mesenteric artery, and coeliac axis were ligated. Unclosed ligatures were then placed about the superior mesenteric artery and the portal vein. These ligatures were now closed simultaneously. In this way the circulation was discontinued in the principal abdominal viscera, including the stomach, intestines, spleen and liver. The dogs were then given intravenous peptone injections. Typical data thus obtained are shown in table 2.

From this table it is seen that marked peptone reactions take place in eviscerated dogs. The fall in arterial blood pressure, however, is less pronounced than in intact dogs, and even less pronounced than in dehepatized dogs. There is therefore undoubtedly a contributory reacting factor in the extra-hepatic abdominal viscera, presumably in the intestines. Almost no recovery is noted in eviscerated dogs.

TABLE 2

*Typical peptone reaction in eviscerated dogs*

Intravenous injections of 0.25 gram Witte's peptone (B) per kilogram of body weight. Morphine-ether anesthesia

| ANIMAL TESTED | CAROTID BLOOD PRESSURE | | | | | | |
|---|---|---|---|---|---|---|---|
| | Initial | 90 seconds | 5 minutes | 10 minutes | 20 minutes | 40 minutes | 60 minutes |
| | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg | *mm.* Hg |
| Eviscerated dog | 130 | 70 | 50 | 48 | 48 | 56 | 50 |
| Control dog | 125 | 32 | 23 | 25 | 40 | 70 | 100 |

## CONCLUSIONS

We conclude from these tests that the reacting tissues in canine peptone shock are widely distributed throughout the body. Canine anaphylactic shock and canine peptone shock therefore cannot be regarded as physiologically identical reactions. This confirms our earlier conclusion based on a study of the refractory periods in the two shocks (3).

## SUMMARY

1. Marked fall in arterial blood pressure is produced by intravenous injections of peptone into dehepatized dogs.

2. Canine peptone shock and canine anaphylactic shock therefore cannot be regarded as physiologically identical reactions.

3. Recovery from peptone shock does not take place in dehepatized dogs.

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# SEROLOGICAL FACTORS OF NATURAL RESISTANCE IN ANIMALS ON A DEFICIENT DIET

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Study of the changes in experimental animals brought about by dietary deficiencies has been directed almost exclusively to physiological and anatomical manifestations. The effects which such diets may have upon serological or immunological properties have received but little attention in spite of the fact that there is abundant clinical evidence associating defective diet with decreased resistance. In numerous publications of a clinical nature a direct correlation has been pointed out between a deficient diet and morbidity and mortality rates from intercurrent infection. Such findings obviously suggest that under conditions of inadequate nutrition the efficiency of the mechanism of resistance is impaired. In so far as can be determined, the connection between these two conditions has not been submitted to direct experimental proof, either with the clinical material suggesting such a relationship or with experimental animals in which nutritional disturbances were artificially induced.

Two papers have appeared which, dealing with the subject from an experimental point of view, seem to have a bearing upon the question. The first of these, that of Hektoen (1), only indirectly touches the point at issue, and the results are inconclusive. Hektoen reports experiments designed to determine the ability of normal and deficient rats to produce heterogenetic antibody. No difference between the two sets of animals could be found, and it must be said that were a deficient animal to show any deviation from normal, that condition would hardly become apparent within the short time (five days) that the

animals were upon the inadequate diet. Zilva (2) in a more recent communication, approached the subject from much the same point of view and measured the production of agglutinating and complement fixing antibodies for *B. typhosus*. In addition Zilva performed repeated titrations for complement. He considers the experimental data as warranting the conclusion that although growth conditions were profoundly modified by the diets supplied, the antibody response to typhoid antigen remained unaffected except in certain animals which had received diets poor in phosphorus. Furthermore, no significant change in the complementing activity of the sera of the animals on scorbutic diet as compared with normal control animals could be detected. In the discussion, however, he specifically points out that the capacity of the body to produce antibody of this type bears no essential relationship to the ability of the body to resist or withstand disease.

In our work we have not been concerned with the ability of the animals, normal and deficient, to elaborate antibody to a specific injected antigen, but have simply been interested in ascertaining a measure of the natural resistance enjoyed by normal rats as compared with those maintained upon an incomplete diet. For this purpose three factors were taken as giving an index of natural resistance—complementing activity of the serum, bactericidal action of the serum, and the ability to stimulate phagocytosis with heterologous leukocytes. It is not suggested that such determinations will yield results to be interpreted as expressing natural resistance, yet it appeared that such factors might be a part in the phenomenon and might afford some information on the subject.

The animals upon which this report is based were rats[1] which had been upon a diet known to be rickets-producing and in which the phosphorus and fat-soluble *A* content was low. Previous to our serologic tests they had been fed exclusively upon this diet for 64 days, and they presented the anatomic pictures already described in the report of Park and his associates (3). In brief, these authors found that while all of these animals had been

[1] Placed at our disposal by Dr. E. A. Park and Dr. G. F. Powers.

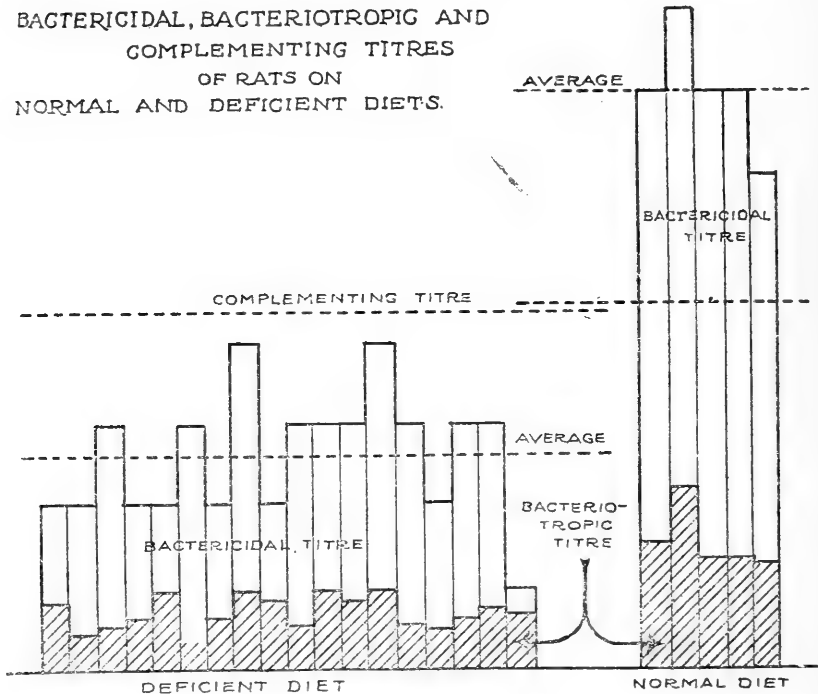
upon the same diet, only those which had not been exposed to radiation from a mercury vapor quartz lamp showed evidence of rickets. Rats treated with the radiation failed to present such a condition. Both of these sets of animals were examined serologically. For purposes of control, normally fed animals of similar weight were used. Previous experiments had shown that while certain differences in result might be expected in rats of different weight and age groups, within any certain group fairly constant and comparable results could be expected.

With the sera of these animals, the blood being taken aseptically from the jugular vein, complement titrations were made in an antisheep system, bactericidal action was determined for *B. typhosus,* and the bacteriotropic activity for *Staphylococcus aureus* with human leukocytes and guinea-pig complement was estimated. It should be noted that all sera from the control as well as from the test animals were subjected to the same treatment, that all titrations were made upon the same day, and that all reagents employed except sera were common to the tests made with both groups of rats.

The titrations for hemolytic complement were performed with the freshly drawn sera diluted 1:20 and combined with 2 units of amboceptor and 0.5 cc. of a 2.5 per cent suspension of sheep cells. Incubation was at 37.5°C. for thirty minutes and the readings that were taken immediately after incubation were confirmed by further readings made after sedimentation of the cells. The results of the complement titration, as indicated upon the graph, do not show the titre for each individual animal. Merely the averages for both groups are presented. While some variation was found in the titers for animals within each group, the range of values was essentially the same and when averaged no significant difference could be detected between the control and the test group.

In the bacteriotropic tests the rat sera were inactivated at 56°C. for thirty minutes. To measured quantities of each serum a constant amount of guinea pig complement was added. Mixtures of complemented sera, human leukocytes, and *Staphylococcus aureus* suspension were made, incubated for 15 minutes, and

spread for staining. In making the counts, both the Wright and the Simon and Lamar indices were recorded. In spite of the well recognized difficulties attending determinations of this type and of interpreting results, certain fairly constant differences appear. The graph presents the results secured by the Wright method, showing the values obtained with each individual animal. It is obvious that the values for the normal animals




are uniformly higher than those for the test rats. Indeed, the lowest index secured in the normal rats is definitely higher than the highest indices found in the deficient animals.

Titrations for bactericidal action were made by the looped pipette method of Wright, the test organism being an 18 hour broth culture of *B. typhosus*. Incubation of serum and culture dilution was allowed to proceed at 37.5°C. for three hours, at the end of which period the mixture was taken up into dextrose

Andrade broth and incubated 24 hours before readings were made. The results secured in these tests are graphically expressed showing the titer for each rat. The individual variation within the two groups was considerable, but, as in the case of the bacteriotropins, the sera of the normal rats were more potent; the average titer for the control group was more than twice as high as the average for the deficient set.

## CONCLUSIONS

The sera from the animals which had been maintained upon a rickets-producing diet show certain differences when compared with sera from the normally fed animals. The outstanding feature is the marked difference in the bactericidal titers; values for the most active sera among the rachitic rats being well below the lowest values secured for the normal animals.

The complement titrations show but little variation, and there appears to be little if any correlation between complementing activity and the bactericidal values of the serum.

The phagocytic indices, while subject to considerable variation within each group, appear in general somewhat lower among the animals fed upon the deficient diet.

It is of particular interest that these serological titrations upon the group of rats which had been upon a deficient diet failed entirely to show any consistent difference which could be correlated with the anatomic findings reported by Powers (3). Both groups, those which actually showed rickets and those which, because of radiation, failed to show rickets, were essentially alike in regard to the reactivity of their sera.

It is not suggested that these changes may be fundamental or that they may be directly associated with the defective diet. It is indeed much more probable that they are indirect, being dependent upon a modified cellular activity induced by failure to provide a proper regimen.

Observations quite similar to these have been made upon a large series of rats[2] which have been upon diets defective in

[2] Placed at our disposal by Dr. L. B. Mendel.

various respects. In not all cases have the results been comparable to those here reported, but the general outcome of the experiments has been quite analogous to the findings in this particular series.

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# BIOLOGICAL REACTIONS OF X-RAYS

## EFFECT OF X-RAYS ON THE RATES OF SPECIFIC HEMOLYSIS[1]

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It has been observed by one of us (1) that the complement content of the blood from patients after x-ray treatment increases in certain instances. A similar result could never be obtained by radiating the blood from the same patient in test tubes. This observation led to the study of the effect of x-rays on the whole hemolytic system outside of the body, with the result that the radiated system hemolyzed more rapidly than the non-radiated controls.

It is difficult to explain this observation with the few facts definitely known about specific hemolysis. Though conceivable as a purely physical phenomenon, the general conception is that some type of chemical reaction is also involved during the hemolytic process. This view depends, of course, to a great extent upon certain properties of the complement, about which a great deal of conflicting literature has been published. The majority of investigators believe that the complement behaves primarily as a ferment (2). It is this interpretation of the nature of the complement that would seem to indicate that hemolysis is largely a chemical reaction. Assuming this to be the case, it is reasonable to suppose that the change in the amount of energy introduced into the system in the form of x-rays, influences the velocity at which equilibrium of the reaction is reached. That this is the case is shown by the following experimental data.

[1] Presented at the October Meeting of the Western Branch of the Society of Experimental Medicine and Biology at Geneva, N. Y.

## EXPERIMENTAL PART

### 1. *Description of reagents*

*Complement.* Guinea-pigs and human complement was obtained by the centrifugation of clotted blood. The serum was immediately removed and preserved at ice temperature.

*Amboceptor.* The product manufactured by the Lederle Antitoxin Company was used in a concentration three times that required to completely hemolyze the amount of blood used.

*Corpuscles.* Fresh sheep cells obtained from a local packing house were washed thoroughly and diluted with physiological salt solution to a 2.5 per cent suspension. Care was taken to centrifuge for the same length of time in all cases so as always to obtain an approximately equal concentration of cells.

### 2. *Description of apparatus*

The rate of hemolysis was determined in small test tubes of approximately the same diameter and thickness of walls (10 cm. x cm.). Mixing of the liquid was accomplished by means of glass rods, the ends of which were bent at right angles.

In all experiments a Kelly Koett x-ray machine equipped with a Coolidge tube was used. This tube (20508) gave an erythema dose, on the human skin, without a filter in two minutes at a distance of 21 cm., 126 K.V., and 10 M.A. The 2.4 cm. aluminum filter gave an erythema dose in four minutes at a distance of 21 cm., 126 K.V., 5 M.A.

### 3. *Method*

*Sensitation of sheep cells.* It was found in our preliminary experiments that unless the amboceptor and corpuscles were uniformly mixed two sets of hemolytic systems did not come to the point of complete hemolysis at the same time. According to our experience, it was impossible to obtain equal distribution of corpuscles and amboceptor by ordinary hand mixing. The best result could only be attained by adding the amboceptor as rapidly as possible to the corpuscles which were stirred by a high

speed agitator. The speed, of course, must be below the breaking point of the cells. By this procedure equal quantities of amboceptor and corpuscles were mixed giving a final concentration of 1.25 per cent cells.

*Determination of the rate of hemolysis.* Into each of the small test tubes was pipetted one cubic centimeter of the diluted complement and one cubic centimeter of the sensitized corpuscles, making a final volume of 2 cc. The stirring rods were then introduced and the contents of each tube mixed by hand at the same speed until complete hemolysis was reached. This point of complete hemolysis was always reached at almost the same length of time (the greatest variation being 30 seconds) in series of four control tubes. A tube of completely hemolyzed blood of the same dilution of complement and volume as the system under examination was used for the comparison of end-points.

To determine the influence of x-rays on the rate of hemolysis two series of tubes containing the same dilution of complement were prepared, the sensitized cells being added to both series as quickly and as nearly simultaneously as possible. The contents of the tubes were then mixed by shaking gently and one series was placed under the Coolidge bulb. Since the absorption of x-rays varies with the depth of the liquid exposed, the tubes were always placed at the same angle (nearly horizontal). The other series of tubes was placed at the same angle as the radiated ones, but protected from the action of the x-rays. The temperature of both series was recorded and only those experiments were used in which the temperature checked within 0.5°. After the completion of the radiation period the stirring rods were introduced and both series of tubes stirred uniformly and continuously until the end-point was reached.

## EXPERIMENTAL DATA

### *Radiation without a filter*

In these experiments a special regulation of the temperature was necessary since the tubes showed a considerable increase in temperature during the radiation period. A satisfactory regula-

tion was obtained by sending over the tubes with an ordinary fan, a current of cold air generated from a properly adjusted pile of ice.

## DISCUSSION OF RESULTS

Our experimental data conclusively shows that the rate at which our hemolytic system went into complete solution was

TABLE 1

*May 10, 1922. Fresh guinea-pigs' complement: titre 0.01. Radiation: No filter —40 cm.; 4 M.A.: 141 K.V. Temperature: 24.5° ± 0.5°C. Average interval between addition of sensitized corpuscles and beginning of radiation was 60 seconds.*

| DILUTION OF COMPLEMENT | RADIATED SERIES | | | NON-RADIATED | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Time of complete hemolysis at radiation periods of | | | Time of complete hemolysis | | | Increase of rate of hemolysis by radiation of | | |
| | 4 minutes | 8 minutes | 12 minutes | 4 minutes | 8 minutes | 12 minutes | 4 minutes | 8 minutes | 12 minutes |
| | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* |
| 0.04 | 12.5 | 12.5 | 10 | 15.5 | 15.5 | 15.0 | 3 | 3 | 5.0 |
| 0.04 | 12.5 | 12.5 | 10 | 15.5 | 15.5 | 15.0 | 3 | 3 | 5.0 |
| 0.02 | 20.5 | 21.5 | 23 | 23.5 | 29.5 | 29.5 | 3 | 8 | 6.5 |
| 0.02 | 20.5 | 21.5 | 23 | 23.5 | 29.5 | 29.5 | 3 | 8 | 6.5 |

Radiation with a filter (alumumum 2.4 mm.)

TABLE 2

*May 18, 1922. Fresh guinea-pigs' complement. Radiation 25 cm.: 4 M.A.: 141 K.V. Filter: Aluminum 2.4 mm.* Temperature: 22.5 plus 0.5°C.

| DILUTION OF COMPLEMENT | RADIATED SERIES | | | | CONTROL SERIES | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time of complete hemolysis at radiation periods of | | | | Time of complete hemolysis | | | | Increase of rate of hemolysis by radiation of | | | |
| | 8 minutes | 12 minutes | 16 minutes | 20 minutes | 8 minutes | 12 minutes | 16 minutes | 20 minutes | 8 minutes | 12 minutes | 16 minutes | 20 minutes |
| | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* | *minutes* |
| 0.04 | 13 | 14 | | | 14 | 16.5 | | | 1 | 2.5 | | |
| 0.04 | 13 | 14 | | | 14 | 16.5 | | | 1 | 2.5 | | |
| 0.02 | 24 | 29 | 29 | 28 | 25 | 32.0 | 36 | 35.5 | 1 | 3.0 | 7 | 7.5 |
| 0.02 | 24 | 29 | 29 | 28 | 25 | 32.0 | 36 | 35.5 | 1 | 3.0 | 7 | 7.5 |

increased by the actions of x-rays. This acceleration took place only when the whole system was exposed to the x-rays, since

radiation of complement alone, sensitized corpuscles alone and complement plus amboceptor alone did not increase the rate of

TABLE 3

*May 11, 1922. Radiation of guinea-pigs' complement. Corpuscles added immediately after radiation period. Stirring then commenced at once. Temperature 23.5°C. Radiation: Same as in table 1 (no filter)*

| DILUTION OF COMPLEMENT | INCREASE OF RATE OF HEMOLYSIS BY RADIATION OF WHOLE SYSTEM—15 MINUTES | RADIATED COMPLEMENT | CONTROL SERIES |
|---|---|---|---|
| | | Time of complete hemolysis at radiation periods of 15 minutes | Time of complete hemolysis—15 minutes |
| | *minutes* | *minutes* | *minutes* |
| 0.02 | 5 | 20 | 16 |
| 0.02 | 5 | 20 | 16 |

TABLE 4

*May 10, 1922. Radiation of sensitized corpuscles. Radiation: As in table 1 (no filter). Complement added simultaneously to both series after radiation period.*

| DILUTION OF COMPLEMENT | RADIATED SERIES | CONTROL SERIES |
|---|---|---|
| | Time of complete hemolysis after radiation periods of 12 minutes | Time of complete hemolysis after 12 minutes |
| | *minutes* | *minutes* |
| 0.04 | 11 | 11 |
| 0.04 | 11 | 11 |
| 0.02 | 20 | 20 |
| 0.02 | 20 | 20 |

TABLE 5

*July 6, 1922. Radiation of complement plus amboceptor. Corpuscles added simultaneously to both series after radiation period. Radiation: As in table 2 (Aluminum filter 2.4)*

| DILUTION OF COMPLEMENT | TIME OF COMPLETE HEMOLYSIS AFTER RADIATION PERIODS OF 15 MINUTES | TIME OF COMPLETE HEMOLYSIS AFTER 15 MINUTES |
|---|---|---|
| | *minutes* | *minutes* |
| 0.04 | 28 | 28 |
| 0.04 | 28 | 28 |

hemolysis of the system. This is proven by the experimental data shown in tables 3, 4, 5 and 6.

These facts that the radiation of the separate components (tables 4, 5, 6 and 7) involved in our hemolytic system has no effect on the rate of hemolysis, and that both series, radiated and non-radiated, reach the same end-point (complete hemolysins) but at different periods of time indicate that the effect of x-rays have been to increase the kinetic energy of the molecules composing the hemolytic system to such an extent that the reaction has reached completion in a shorter period of time, an effect

TABLE 6

*Radiation of complement alone with a filter (aluminum 2.4 mm.). Complement added simultaneously to both series after radiation period. Radiation as in table 2.*

| DILUTION OF COMPLEMENT | INCREASE OF RATE OF HEMOLYSIS BY RADIATION OF WHOLE SYSTEM | | RADIATED SERIES (COMPLEMENT ONLY RADIATED) Time of complete hemolysis after radiation periods of | | CONTROL SERIES Time of complete hemolysis after | |
|---|---|---|---|---|---|---|
| | 16 minutes | 20 minutes | 16 minutes | 20 minutes | 16 minutes | 20 minutes |
| | | | *minutes* | *minutes* | *minutes* | *minutes* |
| 0.02 | 7.0 | 7.5 | 19 | 19 | 19 | 19 |
| 0.02 | 7.0 | 7.5 | 19 | 19 | 19 | 19 |

TABLE 7

| DILUTION OF COMPLEMENT | RATE OF HEMOLYSIS AT 20°C. | RATE OF HEMOLYSIS AT 30°C. | ACCELERATION OF HEMOLYSIS BY 10° RISE OF TEMPERATURE |
|---|---|---|---|
| | *minutes* | *minutes* | |
| 100 | 11 | 4.75 | 2.3 |
| 60 | 18 | 7.5 | 2.4 |
| 40 | 28 | 10.6 | 2.6 |
| 30 | 40 | 15.0 | 2.7 |
| 20 | 94 | 33.0 | 2.9 |

analogous to that produced in the same system by a rise of temperature (3). This increase in kinetic energy is not the only effect of x-rays on our hemolytic system. There is also (see table 4) a simultaneous slow destruction of the complement taking place, which, if the radiation is intense enough may influence the hemolytic system to a greater extent than the accelerating action due to the increase of the kinetic energy of

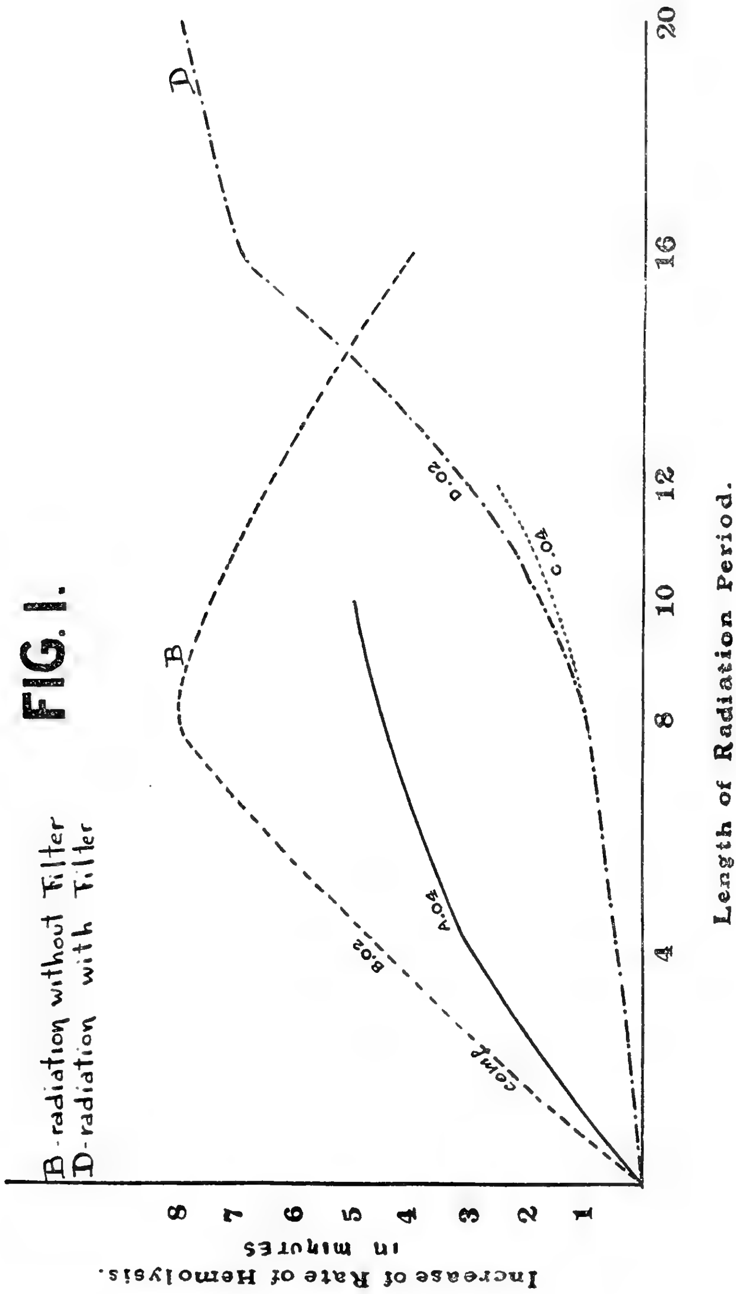
FIG. I.
B-radiation without Filter
D-radiation with Filter
B
D
B.02
A.04
D.02
C.04
Contr
4
8
10
12
16
20
1
2
3
4
5
6
7
8
Length of Radiation Period.
Increase of Rate of Hemolysis.
in minutes

the molecules of our system. This destructive action of x-rays on the complement is strikingly shown in table 4 where radiation of the whole system increased the rate of hemolysis five minutes, while simultaneous radiation of the same dilution of complement alone decreased the rate of hemolysis by four minutes. This destruction of the complement was observed only in those experiments in which no filters were employed. The same facts are graphically represented in figure 1. It will be noted that the slope of curve B, where no filter was used, continuously decreases after 8–9 minutes radiation, while the slope of curve D, where an aluminum filter was employed, decreased only slightly, even after sixteen minutes' radiation.

On further reviewing the data it is observed that there is no direct relationship between the dilution of the complement and the time of complete hemolysis. The lower dilutions (one-half the higher) always hemolyze more rapidly than calculation would require (see tables 1 and 2). Furthermore, as portrayed in figure 1 (see also table 1) the lower dilutions of complement on radiation show a greater increase in their rate of hemolysis than the higher ones. This variation of velocity of hemolysis with concentration takes place both with and without a filter, but it is much more noticeable in the latter case. Here again, we find a similarity between the influence of radiation and rise of temperature on a hemolytic system. Thus, on calculating the data of Kiss (4), we find that at a concentration of 100 units of complement an increase of 10° accelerated the rate of hemolysis 2.3 times, while at a concentration of 20 units the velocity was increased to 2.9 times. This relationship is tabulated in table 7.

## SUMMARY

1. The radiation of the individual components of a hemolytic system does not increase the rate of hemolysis of that system.

2. Radiation of the whole hemolytic system increases the rate of hemolysis of that system.

3. X-rays influence the velocity at which the equilibrium of a reaction is reached. Further studies are in progress to determine the effect of x-rays on the velocity of chemical reactions.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## V. MECHANISM OF THE INCREASED HEPATIC RESISTANCE DURING CANINE PEPTONE SHOCK

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One of the important physiological factors in canine peptone shock is the increased resistance to blood flow through the liver (1). We have endeavored to determine the mechanism of this increase by histological methods.

To prepare material for this study, livers were fixed by perfusion methods. By the preferable technic, a canula is tied in the pancreatic branch of the portal vein, and connected with constant-pressure bottles containing warm (38°C.) Ringer's solution and warm Kaiserling's solution. An unclosed ligature is then placed about the mesenteric vein and hepatic artery. The dog is now thrown into shock by an intravenous peptone injection. At the desired stage of the shock, the Ringer's solution clamp is opened, the abdominal vena cava cut, the mesenteric-hepatic ligature tightened, and the thoracic vena cava cut.

After 350 to 500 cc. Ringer's solution has passed through the liver, washing out the easily removable blood, the perfusion is changed to Kaiserling's solution. About 1500 cc. Kaiserling's solution is allowed to flow through the organ. This is followed without change of pressure by 2000 cc. warm non-acid Zenker's solution containing 10 per cent formalin. The formalized Zenker's solution is used because it immediately hardens the liver, so that practically no hepatic collapse takes place on stopping the

perfusion. The hardened liver is now removed, and emersed in Kaiserling's solution. Samples are cut for histological study twenty-four hours later.

By an alternate method, the liver is perfused through a canula tied in the portal vein. This method, however, has the disadvantage of temporarily interrupting the hepatic circulation.

The routine pressure used in these perfusions was 15 to 18 mm. Hg, the approximate portal pressure during peptone shock (1). Certain livers, however, were fixed at 8 to 10 mm. Hg, the approximate normal portal blood pressure in dogs, others at pressures as high as 40 mm. Hg.

The results here reported are based on a study of twelve peptone shock livers and eight normal controls. The majority of the shock livers represent the eight to twelve minute stage of the peptone reaction. Livers, however, were studied as early as the second minute of peptone shock, and as late as the thirtieth minute. The histopathological findings may be conveniently presented under three headings: (*a*) Changes in the parenchymatous cells; (*b*) changes in the interstitial tissue fluids; and (*c*) changes in the blood vessels.

## I. PARENCHYMATOUS CHANGES

All peptone shock livers thus far studied have shown marked swelling and vacuolization of the parenchymatous cells. These changes are similar to the changes described by Weil and Eggleston (2) in canine anaphylaxis. This parenchymatous change is least marked in the earlier stages of peptone shock, when it may be confined to certain lobules or to certain portions of each lobule. In the later stages of the reaction, marked swelling and vacuolization is seen in practically all parenchymatous cells.

## II. LYMPHATIC CHANGES

All peptone shock livers thus far studied have shown marked increases in the amount of the interstitial tissue fluids, and marked changes in the nature of these fluids. The increase is shown by: (*a*) Edema of the connective tissue structures, particularly of the adventitia of the hepatic veins; (*b*) dilatation

and even rupture of the formed lymphatics; and (*c*) mechanical separation of the sinusoidal endothelium from the parenchymatous cells.

Lymphatics are more numerous in the liver than we had previously supposed. The smaller hepatic veins are often paralleled by from four to eight lymph channels, each channel having a potential diameter greater than that of the accompanying vein. The separation of the sinusoidal endothelium from the parenchymatous cells is usually eccentric, the partially collapsed capillary being attached at one side to the sinusoidal wall. Occasionally, however, a concentric separation is observed.

Changes in the composition of the interstitial tissue fluids are shown by: (*a*) Altered staining reactions; and (*b*) the presence of extravasated red blood corpuscles. The contents of the lymphatics usually stains pink with eosin, giving the appearance of colloid. Numerous extravasted red blood corpuscles are always found, particularly in the edematous adventitia of the hepatic veins. These changes are in line with the well known changes in the lymph collected from the thoracic duct during peptone shock. This lymph is markedly increased in amount, its specific gravity is increased, it is highly coagulable, and contains numerous red blood corpuscles.

## III. VASCULAR CHANGES

All peptone shock livers thus far studied have shown marked vascular changes. The changes are:

*a. Sinusoidal vasoconstriction.* This is shown by a marked narrowing, or even complete collapse of the sinusoidal capillaries, with or without separation from the sinusoidal parenchyma. We believe the main factor causing this narrowing is increased tissue pressure from perivascular edema and parenchymatous swelling. Whether or not there is in addition an active capillary vasoconstriction cannot be determined by our methods.

*b. Hepatic venoconstriction.* A similar narrowing is usually observed in the hepatic veins. The portal veins are apparently not constricted. We believe the main factor causing this narrowing is increased tissue pressure from perivascular edema and

perivascular lymphatic dilatation. Whether or not there is in addition an active hepatic venoconstriction cannot be determined by our method. We believe, however, the evidence is against this factor, since venoconstriction cannot be demonstrated by hepatic perfusion with adrenalin and barium chloride (3).

*c. Sinusoidal stasis.* With the routine perfusion pressure, the blood is always incompletely removed from the peptone shock livers. The retained blood is in the form of sinusoidal plugs, with corpuscles occasionally so closely packed together that it is difficult to determine the outlines of the individual cells. This finding confirms the observation of Simons (4), who found remnants of sinusoidal plugs in the livers of dogs killed eight and fifteen days after multiple intravenous peptone injections.

The amount of sinusoidal stasis depends upon the stage of the peptone reaction. In the early stages of the shock, the majority of the sinusoids may be occluded by corpuscle plugs, particularly toward the centers of the lobules. In the later stages, only an occasional sinusoidal plug may be noted.

The sinusoidal plugs show no evidence of fibrin, from which we conclude that their formation is not due to local coagulation. If defibrinated whole blood is mixed with peptone in the relative proportions employed in the production of peptone shock, no hemagglutination is observed, and no increased adhesiveness of the corpuscles on centrifugation. We therefore believe that hemagglutination plays no rôle in the production of the sinusoidal stasis.

*d. Venous stasis.* The portal veins are always free from corpuscles in our preparations. The hepatic veins are usually free from corpuscles. Occasionally, however, a considerable number of narrowed hepatic veins are found plugged with corpuscle masses.

*e. Leucocytic deposits.* Peptone shock livers always show numerous leucocytes adherent to the sinusoidal walls. The number of adherent leucocytes is greatest in the later stages of the shock, when occasional sinusoidal occlusions with leucocytic masses are seen.

## IV. PHYSIOLOGICAL DEDUCTIONS

From the above findings we believe the dominant factor producing increased resistance to blood flow through the liver

during canine peptone shock, is a suddenly increased sinusoidal permeability. This produces: (*a*) An explosive hepatic edema and parenchymatous swelling, increasing tissue pressure sufficient to cause passive sinusoidal vasoconstriction and passive hepatic venoconstriction; and (*b*) a sudden loss of plasma from the sinusoidal blood, increasing local blood viscosity sufficient to cause temporary sinusoidal stasis and even stasis in the narrowed hepatic veins. A minor factor operative in increasing this resistance is, (*c*) sinusoidal narrowing from leucocytic deposits.

The above factors, of course, may possibly be augmented by an active vasoconstriction. We have no experimental evidence, however, in support of this factor.

## V. SUMMARY

1. The dominant factor increasing resistance to blood flow through the liver during canine peptone shock is a suddenly increased permeability of the sinusoidal endothelium.

2. This produces an explosive hepatic edema accompanied by swelling of the parenchymatous cells, increasing local tissue pressure sufficient to cause passive sinusoidal vasoconstriction and passive constriction of the hepatic veins.

3. The sudden loss of fluid from the sinusoidal blood increases local blood viscosity sufficient to cause temporary sinusoidal stasis, and even stasis in the narrowed hepatic veins.

4. A minor factor contributing to this increased resistance, is sinusoidal narrowing from leucocytic deposits.

5. We have no evidence thus far that active hepatic vasoconstriction is a factor in this reaction.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## VI. HISTAMINE REACTIONS IN ISOLATED CANINE TISSUES

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The sudden fall in arterial blood pressure, the characteristic feature of acute anaphylactic shock in dogs, is due to hepatic reactions (1). We believe these reactions consist in part at least in the sudden formation or liberation of hepatic products having histamine-like effects on the extra-hepatic blood vessels (2). As a basis of comparison we have studied histamine reactions in various isolated canine tissues. The results thus far obtained will be presented under two headings: First, topographical distribution of the histamine-reacting tissues in the animal body, and second, histamine reactions in blood-free perfusions of isolated organs. Defibrinated blood perfusions will be reported later.

### DISTRIBUTION OF HISTAMINE-REACTING TISSUES

*1. Intact dogs.* Histamine shock in intact dogs is characterized by a sudden, pronounced fall in arterial blood pressure. This fall begins within ten seconds after commencing the histamine injection. In this feature histamine shock differs from anaphylactic shock, in which the fall in arterial pressure is always delayed at least forty seconds.

In histamine shock the blood pressure is reduced to about 40 mm. Hg by the end of one minute, and then slowly sinks to a minimum of about 25 mm. Hg by the end of five minutes. The pressure then very slowly rises, reaching normal in from forty to ninety minutes, depending upon the histamine dose injected.

*2. Dehepatized dogs.* The dehepatization tests were made by the technic previously described (3). Intravenous injections of 1 to 3 mgm. of histamine per kilogram of body weight produce reactions in dehepatized dogs apparently identical with those in intact dogs. Recovery takes place as promptly and completely as in intact animals.

The liver, therefore, is not an essential or dominant organ in the production of canine histamine shock, nor is it essential to histamine recovery. In this respect histamine shock differs from canine peptone shock, in which recovery does not take place in dehepatized dogs (3).

*3. Eviscerated dogs.* The evisceration tests were made by the technic previously described (3). Reactions apparently identical with those in intact dogs are produced by intravenous injections of histamine into eviscerated dogs. Recovery is normal. In this respect, also, histamine shock differs from canine peptone shock, the severity of the peptone reaction being markedly reduced in eviscerated dogs (3).

*4. Conclusion.* We conclude from these tests that the essential or dominant reacting tissues in canine histamine shock are either confined to the extra-hepatic and extra-intestinal parts, or as seems probable from isolation tests (see below) are fairly evenly distributed throughout the body as a whole.

## REACTIONS IN ISOLATED CANINE ORGANS

Reactions in isolated organs were tested by the perfusion technic previously described (4). The following organs have thus far been studied:

*1. Hind quarters.* Rapid decrease in perfusion resistance, increasing the rate of perfusion flow from 15 to 200 per cent depending upon the initial vascular tone (*A*, fig. 1). The reaction reaches its maximum in about two minutes. Marked edema of the hind quarters.

*2. Intestines.* Gradual increase in perfusion resistance, reducing the rate of perfusion flow from 40 to 60 per cent, depending upon the histamine dose used (*B*, fig. 1). The reaction reaches

its height in about five minutes. Marked peristaltic movements. Peritoneal transudation. Rapid filling of the intestinal lumen with fluid.

*3. Liver.* Rapid increase in perfusion resistance, reducing the rate of perfusion flow from 10 to 90 per cent, depending upon the histamine dose used (*C*, fig. 1). The reaction reaches its height in about two minutes. Peritoneal transudation.

*4. Lungs.* Rapid increase in perfusion resistance, reducing the rate of perfusion flow from 60 to 90 per cent, depending upon the histamine dose used (*D*, fig. 1). The reaction reaches its

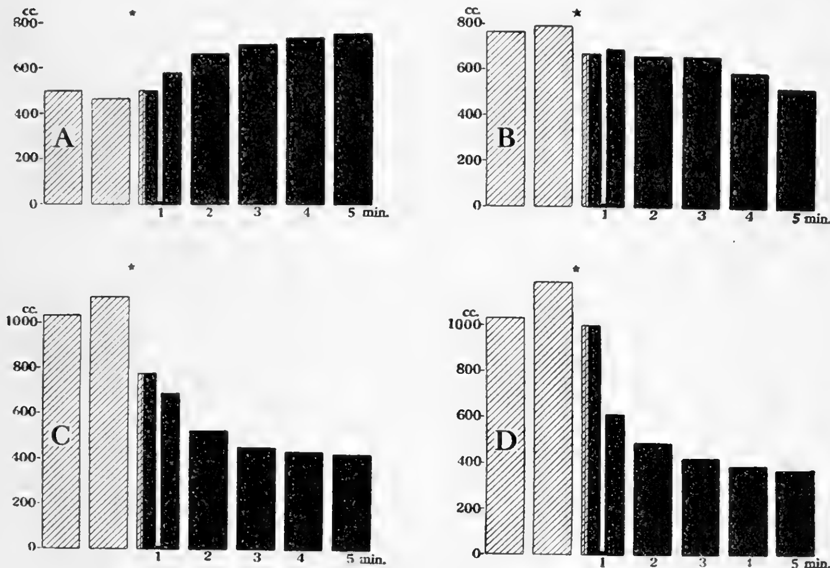


Fig. 1. Histamine Reactions in Isolated Canine Organs

Cross-hatched areas show perfusion flow per minute with Locke's solution. Black areas show rate per minute (or half-minute) with Locke's solution plus histamine. Composite picture in each case of two perfusions with 10 and 30 mgm. histamine respectively per 1000 cc. Locke's solution, corresponding roughly to intravenous injections of 1 and 3 mgm. per kilogram of body weight. Stars show time of changing perfusion clamps.

*A* = Hind-quarters: Perfusion pressure 80 to 100 mm. Hg.

*B* = Intestines: Afferent perfusion pressure, 80 to 100 mm. Hg; efferent perfusion pressure, 10 mm. Hg (normal portal pressure).

*C* = Liver: Perfusion pressure, 18 mm. Hg (portal pressure during histamine shock).

*D* = Lungs: Perfusion pressure, 25 mm. Hg.

height in about two minutes. During this reaction the lungs take on a rubber-like consistency. Incomplete pulmonary collapse with the escape of large amounts of frothy fluid from the trachea on releasing the tracheal clamp. Pleural transudation.

5. *Kidneys.* Rapid increase in perfusion resistance, reducing the rate of perfusion flow from 15 to 50 per cent, depending upon the histamine dose used. The reaction reaches its height in about three minutes. Peritoneal transudation. Increased urine formation.

## CONCLUSIONS

We conclude from the above tests that the dominant factor producing the acute fall in arterial blood pressure in canine histamine shock is probably the suddenly increased permeability of the capillary endothelium. This conclusion is in line with the conclusion of Dale and Laidlaw (5).

The distinct vaso-dilation observed in the isolated hind quarters, and the marked vaso-constriction in other parts of the body are in line with previous observations (6, 7). These reactions, however, would tend to neutralize each other, in their effects on arterial blood pressure.

The probabilities are that the marked vaso-constriction observed in blood-free perfusions of the lungs, liver, kidneys and intestines, is due to the explosively increased tissue pressure from edema (8).

## SUMMARY

1. The dominant reacting tissues in canine histamine shock are fairly evenly distributed throughout the body.

2. The most striking reaction in blood-free perfusions of isolated canine tissues is the explosive edema of all organs thus far tested.

3. Reactions of secondary importance are: (*a*) Marked decrease in perfusion resistance in the hind quarters, and (*b*) Marked increase in perfusion resistance in the lungs, liver, intestines and kidneys.

4. We believe this increased perfusion resistance is largely due to increased tissue pressure from edema.

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# ZONE PHENOMENA IN COMPLEMENT FIXATION WITH "RESIDUE" ANTIGENS

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In 1912 Dean (1) showed that the relative proportion of antigen and antibody is of the greatest importance in both precipitation and complement fixation reactions. He showed, conclusively, that to determine quantitatively the antibody content of a serum or the antigen content of a serum or extract, it is necessary to titrate decreasing quantities of antiserum with decreasing amounts of antigen.

The significance of this work does not seem to have been fully recognized although its practical bearing on any experiments dealing with precipitation or complement fixation tests is of the utmost importance. In fact, if the quantitative relation of antigen and antibody is not understood, especially in complement fixation reactions, antibodies in a strong serum or antigen in a strong extract may be entirely missed, or the opposite may occur, that is, antibodies in a weak serum, or antigen in a weak extract, may be overlooked.

The experiments reported in this paper were carried out in connection with a general study on the properties of substances derived from bacteria which have been more fully described in a preceding paper by Zinsser and Parker (2) and spoken of as the "residue" antigens. Although the experiments tabulated below are in principle entirely confirmatory of Dean's work, it seemed important to us to develop them at some length, and to report them, both because of their bearing on the value of the "residue" antigens for serological purposes and because of their emphasis upon the importance of Dean's observations. Since most of the observations on the zone phenomena were done with complement fixation reactions, we include this part of the work only in the present report.

The sera used were obtained from rabbits which had been immunized to pneumococcus I, the Rawling's strain of typhoid bacilli, and with normal horse serum, respectively. The "residue" antigens employed were prepared according to the method described more fully in the paper referred to above by Zinsser and Parker. Since, as has been mentioned in the paper above referred to, the typhoid bacillus was one of the few from which it was difficult to obtain any considerable quantities of the "residue" material, the typhoid antigen used in experiments 2 and 3 consisted of a filtered neutral salt solution extract of typhoid bacilli. The complement fixation reactions were carried out in the ordinary way with the rabbit anti-sheep hemolytic system and guinea-pig serum as complement. Two units of complement were used unless otherwise noted. Since, as will appear in the text, the order in which the substances are run into the test tubes makes a considerable difference in results, the order in every case is the one given in the tables.

*Experiment 1*

| | PNEUMOCOCCUS RESIDUE | ANTI PNEUMOCOCCUS RABBIT SERUM 153 | COMPLEMENT | | A | B |
|---|---|---|---|---|---|---|
| | | | | | With 2 units complement | With 4 units complement |
| 1 | 0.05 | 0.05 | 2 or 4 units | 1 hour at 37°C. Sensitized cells. Readings after thirty minutes at 37°C. | ++++ | ++++ |
| 2 | 0.025 | 0.05 | 2 or 4 units | | ++++ | ++++ |
| 3 | 0.01 | 0.05 | 2 or 4 units | | ++++ | 0 |
| 4 | 0.005 | 0.05 | 2 or 4 units | | ++++ | 0 |
| 5 | 0.05 | 0.025 | 2 or 4 units | | ++++ | ++++ |
| 6 | 0.025 | 0.025 | 2 or 4 units | | ++++ | ++++ |
| 7 | 0.01 | 0.025 | 2 or 4 units | | ++++ | ++++ |
| 8 | 0.005 | 0.025 | 2 or 4 units | | ++++ | ± |
| 9 | 0.05 | 0.01 | 2 or 4 units | | ++++ | 0 |
| 10 | 0.025 | 0.01 | 2 or 4 units | | ++++ | ± |
| 11 | 0.01 | 0.01 | 2 or 4 units | | ++++ | ++++ |
| 12 | 0.005 | 0.01 | 2 or 4 units | | ++++ | ++++ |
| 13 | 0.05 | 0.005 | 2 or 4 units | | 0 | 0 |
| 14 | 0.025 | 0.005 | 2 or 4 units | | 0 | 0 |
| 15 | 0.01 | 0.005 | 2 or 4 units | | ++++ | 0 |
| 16 | 0.005 | 0.005 | 2 or 4 units | | ++++ | + |

In experiment 1, the tubes were set up in duplicate, the first series of tubes (A) with two units of complement; the second series (B), with four units of complement. Tubes 13 and 14, series A, and 9 and 10, series B, show the inhibition effect of an excess of antigen; tubes 3 and 4, series B, the inhibition effect of an excess of antibody. Under the conditions of this experiment, the optimal proportion of antigen and antiserum seems to have been approximately as 1:1. 0.005 cc. of antiserum and 0.005 cc. of antigen fixed two units, but not four units of complement. Twice this amount of antigen and serum, that is 0.01 cc. fixed twice the amount of complement or four units, and most likely, if the experiment had been carried out, and the amount of complement fixed had not been interfered with, a possible formation of a precipitate, 0.05 cc. of antigen and antibody, would have bound twenty units of complement.

*Experiment 2*

| | TYPHOID ANTIGEN | ANTI-TYPHOID SERUM 150 OR M, OR NORMAL RABBIT SERUM | COMPLEMENT | | WITH ANTI-TYPHOID RABBIT SERUM 150 | WITH ANTI-TYPHOID RABBIT SERUM M | WITH NORMAL RABBIT SERUM |
|---|---|---|---|---|---|---|---|
| | cc. | | | | | | |
| 1 | 0.2 | 0.05 | 2 units | 1 hour at 37°C. Sensitized cells. Readings after 30 minutes at 37°C. | ± | ++++ | 0 |
| 2 | 0.1 | 0.05 | 2 units | | 0 | ++++ | 0 |
| 3 | 0.05 | 0.05 | 2 units | | 0 | ++++ | 0 |
| 4 | 0.01 | 0.05 | 2 units | | 0 | + | 0 |
| 5 | 0.2 | 0.025 | 2 units | | ++++ | ++++ | 0 |
| 6 | 0.1 | 0.025 | 2 units | | 0 | ++++ | 0 |
| 7 | 0.05 | 0.025 | 2 units | | 0 | +++ | 0 |
| 8 | 0.01 | 0.025 | 2 units | | 0 | 0 | 0 |
| 9 | 0.2 | 0.01 | 2 units | | ++++ | 0 | 0 |
| 10 | 0.1 | 0.01 | 2 units | | ++++ | ± | 0 |
| 11 | 0.05 | 0.01 | 2 units | | ± | 0 | 0 |
| 12 | 0.01 | 0.01 | 2 units | | 0 | 0 | 0 |
| 13 | 1.5 | | 2 units | | 0 | | |
| 14 | | 0.05 | 2 units | | 0 | | |
| 15 | | 0.05 | 2 units | | | 0 | |
| 16 | | 0.05 | 2 units | | | | 0 |
| 17 | | | 2 units | | 0 | | |

Experiment 2 compares a weak and a strong antityphoid serum. It is evident from looking at experiment 2 that if 0.05 cc. of the strong serum, 150, had been used in the ordinary way for quantitative determination of antigen, that is, constant amounts of serum with diminishing amounts of antigen, no antigen would have been demonstrated in the typhoid extract. From this it is clear that the strength of an antigen or antiserum cannot be ascertained without titrating falling amounts of anti serum with falling amounts of antigen.

*Experiment 3*

| | RABBIT ANTI-HORSE SERUM | COMPLEMENT | NORMAL HORSE SERUM | | |
|---|---|---|---|---|---|
| | | | *cc.* | | |
| 1 | 0.025 | 2 units | 0.01 | 1 hour at 37°C. Sensitized cells. Readings after 30 minutes at 37°C. | 0 |
| 2 | 0.025 | 2 units | 0.05 | | ± |
| 3 | 0.025 | 2 units | 0.01 | | +++ |
| 4 | 0.025 | 2 units | 0.005 | | +++ to ++++ |
| 5 | 0.025 | 2 units | 0.001 | | ++++ |
| 6 | 0.025 | 2 units | 0.0005 | | ++++ |
| 7 | 0.025 | 2 units | 0.0001 | | ++++ |
| 8 | 0.01 | 2 units | 0.1 | | 0 |
| 9 | 0.01 | 2 units | 0.05 | | 0 |
| 10 | 0.01 | 2 units | 0.01 | | ± |
| 11 | 0.01 | 2 units | 0.005 | | ± |
| 12 | 0.01 | 2 units | 0.001 | | ++++ |
| 13 | 0.01 | 2 units | 0.0005 | | ++++ |
| 14 | 0.01 | 2 units | 0.0001 | | +++ |
| 15 | 0.005 | 2 units | 0.1 | | 0 |
| 16 | 0.005 | 2 units | 0.05 | | 0 |
| 17 | 0.005 | 2 units | 0.01 | | 0 |
| 18 | 0.005 | 2 units | 0.005 | | 0 |
| 19 | 0.005 | 2 units | 0.001 | | 0 |
| 20 | 0.005 | 2 units | 0.0005 | | 0 |
| 21 | 0.005 | 2 units | 0.0001 | | +++ to ++ |
| 22 | 0.025 | 2 units | | | 0 |
| 23 | | 2 units | 0.1 | | 0 |
| 24 | | 2 units | | | 0 |

Experiment 3 demonstrates the delicacy of the complement fixation reaction if carried out under proper conditions. The rabbit antihorse serum was weak. It gave a good ring test only when the antigen was diluted 1:100. This experiment is in-

teresting in showing how small a quantity of antigen or antibody can be demonstrated in a serum. This point may have practical application in work on the presence of specific antibody in the serum of hypersensitive individuals. It is not impossible that the failure of most workers to demonstrate antibodies in the blood of hypersensitive persons by means of complement fixation reactions may be due to the fact that the amount of antigen used in the tests was too large to demonstrate the presence of very minute quantities of antibody. Experiment 3 shows that if 0.1 cc. of the serum of a hypersensitive person had the same antibody content as 0.005 cc. of the rabbit anti-horse serum, no fixation would have occurred unless 0.0001 cc. of normal horse serum had been used in the test.

*Experiment 4*

| | TYPHOID ANTIGEN | RABBIT ANTI TYPHOID SERUM 150 | COMPLEMENT | COMPLEMENT ADDED THREE MINUTES AFTER ANTIGEN AND ANTIBODY | | COMPLEMENT PUT IN TUBE FIRST | |
|---|---|---|---|---|---|---|---|
| | | | | 2 units complement | 4 units complement | 2 units complement | 4 units complement |
| | *cc.* | *cc.* | | | | | |
| 1 | 0.5 | 0.05 | Complement | ++++ | +++ to ++++ | ++++ | ++++ |
| 2 | 0.1 | 0.05 | Complement | + | 0 | ++++ | ++++ |
| 3 | 0.02 | 0.05 | Complement | 0 | 0 | +++ | 0 |
| 4 | 0.5 | 0.025 | Complement | ++++ | ++++ | ++++ | ++++ |
| 5 | 0.1 | 0.025 | Complement | + | 0 | ++++ | ++++ |
| 6 | 0.02 | 0.025 | Complement | 0 | 0 | ++++ | 0 |
| 7 | 0.5 | 0.01 | Complement | ++++ | ++++ | ++++ | ++++ |
| 8 | 0.1 | 0.01 | Complement | ++ | 0 | ++++ | ++++ |
| 9 | 0.02 | 0.01 | Complement | 0 | 0 | ++++ | 0 |
| 10 | 0.5 | 0.005 | Complement | +++ to ++++ | +++ | ++++ | ++++ |
| 11 | 0.1 | 0.005 | Complement | ++++ | ++ | ++++ | ++++ |
| 12 | 0.02 | 0.005 | Complement | 0 | 0 | ++ to +++ | 0 |

All controls negative.

A point which Dean emphasizes in his paper and which we also confirmed, was that in order to get the greatest fixation, the complement must be present when antigen and antibody come together. Experiment 4 demonstrates this point.

## CONCLUSIONS

From the experiments cited above, the following conclusions may be formulated:

1. They confirm definitely the observations of Dean on the great importance of the adjustment of proportionate amounts in determining antigen-antibody reactions.

2. They demonstrate the practical point that astonishingly minute amounts of antibody or antigen may be demonstrated by complement fixation, provided the antigen, or, conversely, the antiserum used in the experiment is employed in a series of diminishing amounts, and, what is of still more practical importance, it shows that unless this procedure is adopted, antigen or antibody which is actually present may be overlooked. This seems to us to have important bearing upon the search for antigen or antibody in conditions of hypersensitiveness in which negative findings are often used as the basis for far-reaching theoretical conclusions.

3. The experiments further confirm the important bearing on results of the sequence in which the various components of the reaction are mixed in the test tubes, the strongest results in complement fixation being obtained of the complement is present from the beginning when antigen and antibody meet.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## VII. QUANTITATIVE CHANGES IN THE HEPATIC PARENCHYMA DURING CANINE PEPTONE SHOCK

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From histological findings previously reported (1), it is evident that there are marked changes in the size of the individual parenchymatous cells of the liver during canine peptone shock, and marked increases in the volume of the interstitial tissue fluids. We have tried to determine the physiological importance of these changes by estimating the resulting increase in net hepatic weight.

To determine the net hepatic weight, the hepatic blood vessels were ligated, and the liver with adjacent portions of the diaphragm removed and weighed. The liver was then perfused with 3000 cc. physiological saline, followed by 2000 cc. distilled water, and the amounts of blood thus removed determined by the hematocrite and colorimetrically. Finally the diaphragm, gall bladder and larger blood vessels were dissected off and weighed. The sum of these weights, together with the estimated weight of the recovered blood, subtracted from the gross hepatic weight, gives the estimated net hepatic weight. A typical example is given below:

| | | |
|---|---|---|
| Weight of dog (composite picture) | | 14.3 kgm. |
| Gross hepatic weight (liver, diaphragm, cannula, etc.) | | 426 grams |
| Diaphragm, gall bladder, cannula, etc. | 88 grams | |
| Blood determined by the hematocrite | 62 cc. | |
| Blood determined colorometrically | 6 cc. | 156 grams |
| Net hepatic weight | | 270 grams |
| Calculated net hepatic weight per kilogram of body weight | | 18.9 grams |

The net hepatic weight thus determined has varied from 15.7 grams to 20.3 grams in our series, an average of 18.9 grams per kilogram of body weight. We have excluded from the series all dogs showing gross pathological lesions.

To determine the changes in net hepatic weight during peptone shock, the dogs were tested in pairs. The dogs of each pair were, so far as possible, dogs of the same age, same breed, and in the same nutritional condition. One dog of each pair was given an intravenous peptone injection, usually 1 gram per kilogram of body weight, about twelve minutes before the hepatic removal. Typical data are shown in table 1.

TABLE 1

*Net hepatic weight during peptone shock*

| BREED | CONTROL DOGS | | | PEPTONE DOGS | | | INCREASE |
|---|---|---|---|---|---|---|---|
| | Weight | Liver | Per kilogram | Weight | Liver | Per kilogram | |
| | *kgm.* | *grams* | *grams* | *kgm.* | *grams* | *grams* | *per cent* |
| 1. Terriers | 11.3 | 227 | 20.3 | 16.1 | 447 | 27.8 | 37 |
| 2. Collies | 12.2 | 192 | 15.7 | 17.3 | 422 | 24.3 | 55 |
| 3. Airdales | 19.5 | 390 | 20.0 | 19.5 | 754 | 39.0 | 95 |

The tests showed an increased net hepatic weight in all peptone shock dogs tested, the increase varying from 37 per cent to 95 per cent, an average increase of 62 per cent in our series. The real increase was undoubtedly greater than this, on account of the error in blood determinations from sinusoidal stasis (1).

The average increased net hepatic weight represents a withdrawal of 11.7 cc. plasma from the circulating blood, per kilogram of body weight. Assuming that a similar withdrawal takes place in other organs, the resulting decrease in blood volume must be an important factor in the production of the characteristic fall in arterial blood pressure.

Attempts to determine the exact amount of the decrease in blood volume by following the variations in the circulating red blood corpuscle count during canine peptone shock are inconclusive. The circulating red corpuscle count is decreased about 10 per cent during the first minute of peptone shock. The blood

count is restored to normal in about six minutes, and increased about 10 per cent above normal by the twelfth minute. An hepatic plasma withdrawal of 11.7 cc. per kilogram of body weight, would, in itself, if unaccompanied by other factors, increase the circulatory red blood corpuscle count about 20 per cent. One of the causes of this discrepancy is a reduction in the number of circulating red blood corpuscles by hepatic sinusoidal stasis.

## SUMMARY

1. The net weight of the liver exclusive of the weight of the contained blood is increased as much as 95 per cent during the early stages of canine peptone shock.

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# HEPATIC REACTIONS IN ANAPHYLAXIS

## VIII. ANAPHYLACTIC REACTIONS IN ISOLATED CANINE ORGANS

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To find a tissue that would serve as a reliable reacting index for the serological analysis of anaphylactic phenomena in dogs, tests were made of anaphylactic reactions in the principal isolated canine organs.

The dogs were sensitized by a subcutaneous injection of 0.3 to 0.5 cc. horse serum per kilogram of body weight, followed two days later by an intravenous injection of the same dose. The isolation tests were made between the seventeenth and twenty-fourth day after the intravenous injection. During this period all control dogs thus far tested have given typical anaphylactic reactions on intravenous injection of horse serum, about a third of them reactions of the fatal type (1). The isolation tests were made by perfusion methods. The perfusion fluid was well-aerated Locke's solution containing 0.04 to 2.5 per cent horse serum. The perfusion technic was otherwise the same as that described in a previous paper (2).

### BLOOD-FREE PERFUSIONS OF ISOLATED ANAPHYLACTIC ORGANS

Distinct anaphylactic phenomena have been demonstrated in all isolated canine organs thus far tested. The following parts have been studied:

*a. Lungs:* Slight preliminary decrease in perfusion resistance, increasing the perfusion rate about 12 per cent (fig. 1). This is followed in from thirty to ninety seconds by a marked increase in perfusion resistance, reducing the perfusion flow fully 70 per

cent. With larger serum doses (A, fig. 1), this reduction reaches its maximum by the end of ninety seconds. With smaller serum doses (B, fig. 1), the maximum is not reached till the fourth to tenth minute.

During the perfusion, the lungs take on a rubber-like consistency. On releasing the tracheal clamp, practically no pulmonary collapse takes place. A large amount of clear, frothy fluid now escapes from the trachea. If the perfusion is continued with the tracheal clamp removed, fluid continues to pour out of the trachea almost as rapidly as it escapes from the efferent cannula.

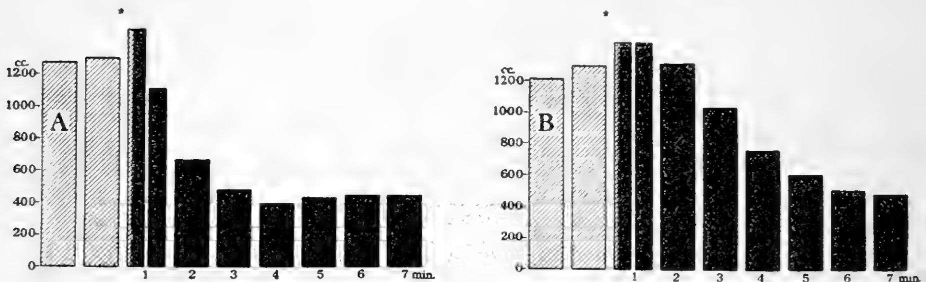


FIG. 1. REACTIONS IN ISOLATED ANAPHYLACTIC LUNGS

Cross-hatched areas show perfusion flow per minute with Locke's solution. Black areas show rate per minute (or half-minute) with Locke's solution plus horse serum. Stars (*) show time of changing the perfusion clamps. Temperature, 38°C., perfusion pressure, 25 mm. Hg.

A = Composite picture of three perfusions with 2.5, 1 and 0.5 per cent horse serum respectively. Control perfusions of normal lungs show no change in perfusion rate within the limits of the experimental error.

B = Composite picture of three perfusions with 0.25, 0.125 and 0.06 per cent horse serum respectively.

On gross section, each large blood vessel of the lungs is seen to be partially collapsed and surrounded by an edematous zone. Whether or not the increased perfusion resistance is due to increased local tissue pressure from this perivascular edema, cannot be determined from our present data. A histological study of these reactions will be reported later.

*b. Intestines:* Gradual increase in perfusion resistance reducing the rate of perfusion flow from 20 to 40 per cent by the end of

seven minutes, depending upon the serum concentration used (fig. 2). Marked peristaltic movements. Increased tone of the intestinal musculature. Distinct edema of the intestinal walls. Peritoneal transudation. Marked increase in the volume of the intestinal contents.

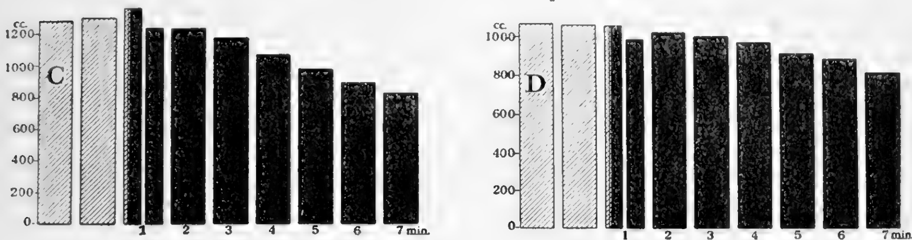


Fig. 2. Reactions in Isolated Anaphylactic Intestines

Afferent perfusion pressure, 80 mm. Hg; efferent perfusion pressure, 10 mm. Hg.
C = Perfusion with 2.5 per cent horse serum.
D = Composite picture of two perfusions with 0.5 and 0.25 per cent horse serum respectively.

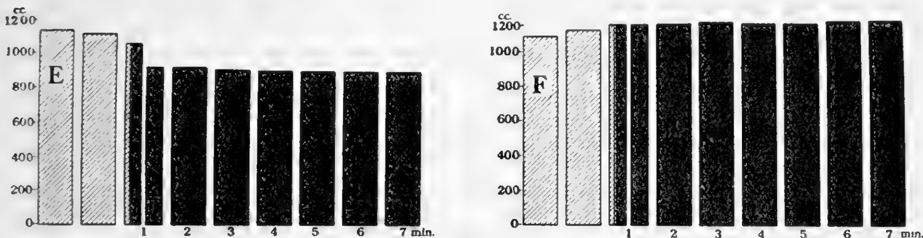


Fig. 3. Reactions in Isolated Anaphylactic Livers

Perfusion pressure, 15 mm. Hg.
E = Composite picture of three perfusions with 2.5, 1 and 0.5 per cent horse serum respectively.
F = Composite picture of two perfusions with 0.25 and 0.125 per cent horse serum respectively.

*c. Liver:* With the larger serum doses (E, fig. 3), an increased perfusion resistance, reducing the rate of perfusion flow about 20 per cent by the end of thirty seconds. With smaller serum doses (F, fig. 3), no demonstrable change in perfusion rate within the limits of the experimental error. Distinct hepatic edema. Peritoneal transudation.

*d. Hind quarters:* On control perfusion of the hind quarters with Locke's solution, the rate of perfusion flow usually increases rapidly during the first two minutes. After the second minute, the flow usually remains fairly constant till the end of the test. The maximum variation during the next seven minutes is usually not more than 5 per cent.

On perfusing anaphylactic hind quarters, two types of reaction have been obtained. Type 1 (G, fig. 4): A decreased perfusion resistance increasing the perfusion rate about 12 per cent by the end of three minutes. No demonstrable edema except a slight edema of the genitalia. Type 2 (H, fig. 4): A rapid increase in

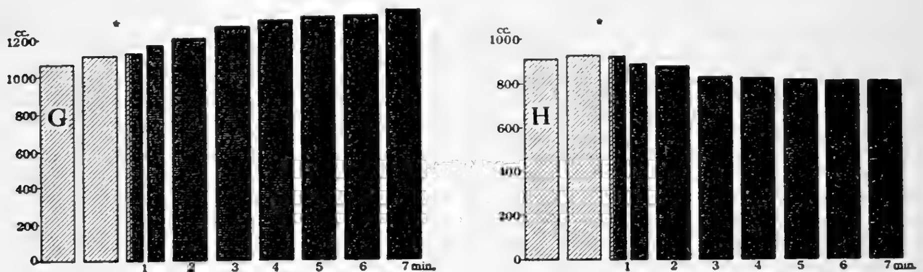


Fig. 4. Reactions in Isolated Anaphylactic Hind Quarters

Perfusion pressure, 80 mm. Hg.
G = Composite picture of two perfusions with 2.5 per cent horse serum.
H = Composite picture of two perfusions with 0.5 and 0.25 per cent horse serum respectively.

perfusion resistance, decreasing the perfusion flow about 10 per cent in from thirty to ninety seconds. Pronounced edema of the hind quarters, particularly of the foot and genitalia.

It is believed that the differences between these two types are merely quantitative, depending upon differences in serum concentration and degree of sensitization. Our data, however, are insufficient to establish this fact.

### COMPARISON WITH HISTAMINE REACTIONS

The above anaphylactic reactions differ quantitatively and in some cases qualitatively from the histamine reactions previously reported (3). For example, the anaphylactic edema

and vasoconstriction in the lungs are much more pronounced than the corresponding histamine vasoconstriction and edema. An initial vasodilation was not observed in our histamine tests.

The intestinal reactions are approximately equal in the two cases.

The anaphylactic reactions in the liver are much less pronounced than the corresponding histamine reactions.

The histamine reactions in the hind quarters are a marked vasodilation with very pronounced edema. These reactions are uniformly more marked than the slight vasodilation and edema in anaphylaxis. Vasoconstriction of the hind quarters was not observed in our histamine tests.

## PHYSIOLOGICAL DEDUCTIONS

We conclude from the above observations that one of the important factors in canine anaphylaxis is an increased specific permeability (susceptibility to specific injury) of the capillary endothelium (4). The capillary endothelium of different parts of the body apparently differs widely in this acquired hyperpermeability. The endothelium of the hind quarters shows the least change from the normal. The hepatic and intestinal endothelium occupy an intermediate position. The pulmonary endothelium shows the most marked alterations.

The vasoconstriction in the different parts of the body varies roughly with the severity of the edema. We believe, therefore, the vasoconstriction is possibly secondary to increased tissue pressure from edema.

Histamine reactions in blood free perfusions of isolated organs differ from histamine reactions in defibrinated blood perfusions (5, 6). No attempt will therefore be made to draw conclusions from the above anaphylactic tests as to the probable mechanism of anaphylactic shock in intact dogs. Defibrinated blood perfusions will be reported later.

## CONCLUSIONS

From these tests it would appear that the only isolated canine organs giving anaphylactic reactions sufficiently pronounced to

be used in the serological analysis of anaphylactic phenomena in dogs are the isolated lungs. Serological analyses by means of the isolated lungs will be reported later.

## SUMMARY

1. The isolated organs of horse serum sensitized dogs, perfused with Locke's solution containing 0.04 to 2.5 per cent horse serum, give the following reactions:

*a. Lungs:* Slight preliminary vasodilation, followed by a pronounced vasoconstriction. Marked edema.

*b. Intestines:* Distinct vasoconstriction. Marked edema.

*c. Liver:* Slight vasoconstriction. Slight edema.

*d. Hind Quarters:* Either: (1) slight vasodilation without edema or (2) slight vasoconstriction with marked edema.

2. The vasoconstriction in these organs may possibly be secondary to increased tissue pressure from edema. If so, increased specific capillary permeability must be looked upon as the dominant underlying physiological factor in these reactions.

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# SOME RELATIONS BETWEEN HYDROGEN ION CONCENTRATION AND THE ANTIGENIC PROPERTIES OF PROTEINS

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## INTRODUCTION

One of the outstanding contributions of recent years to the study of the chemistry and physics of the proteins is the demonstration that the reactions of these substances with electrolytes are capable of stoichiometrical analysis. The work of Jacques Loeb and his collaborators, in this country, and of Sorenson and many others has gone far in removing some of these reactions from the fields of more empirical colloid chemistry into the realms of classical chemistry. They have indicated certain important possibilities in the quantitation of the dynamics of systems containing proteins. It is not possible, nor is it desirable, to review this work here. It is perhaps sufficient to refer to the original papers by Loeb and others in the Journal of General Physiology (1918, to date) and to the concise summary in the recent monograph by Loeb (1922) and to the review by Bancroft (1922). The significance of these contributions in the fields of bacterial physiology have already been reviewed by one of us (Falk, 1923) and have been subjected to experimental application by Winslow and Falk (1923, 1923 a). It is our purpose here merely to review a few outstanding facts which are essential to

[1] The studies reported here were aided by a grant from the Loomis Research Fund of the Yale School of Medicine.

[2] Preliminary reports of this work have been presented before the Society of American Bacteriologists (Abstr. Bact., January, 1923) and the Society for Experimental Biology and Medicine, Proceedings for January 17, 1923.

the understanding of the hypotheses upon which the work reported here was developed.

It is quite clearly established that when proteins are dissolved in water (using the term "dissolved" to apply to true solution, colloidal suspension and all the intermediate stages between the two which are known to occur with little or no discontinuity) they are electrically charged in the same sense in which ions are electrically charged. It is also established that by appropriately modifying the hydrogen-ion concentration of protein solutions the electrical charge on the protein can be modified qualitatively as well as quantitatively and an electro-positively charged protein can be converted into an electro-negatively charged protein and vice versa. By appropriate addition of acid or alkali, the protein can be obtained in the intermediate condition in which it is without electrical charge. When the protein is without electrical charge it is said to be *iso-electric* with the solvent and the hydrogen-ion concentration of the solution is referred to as the *iso-electric point.* For detailed discussion of these physico-chemical characteristics of proteins, we must refer to Loeb (1922) and to the bibliography which he has compiled. The recent contribution of Thomas and Kelly (1922) may be consulted for a table of the iso-electric points of some proteins. The evidence that such properties of protein solutions as osmotic pressure, swelling power, viscosity, power to combine with ions, electrostatic charges, etc., are functions of the hydrogen-ion concentration of the solutions cannot be doubted. Acquaintance with these recent contributions led us to raise the following questions. Does change of the hydrogen-ion concentration modify the antigenic characteristics of the protein at the same time that it produces effects upon their physico-chemical properties? Are any changes which occur merely quantitative changes in antigenic potency, or are they qualitative as well?

It is perhaps pertinent to mention here that the physico-chemical properties of proteins may be very markedly affected within ranges of hydrogen-ion concentration which are without important hydrolytic effects upon the protein molecules and that such changes in the protein are reversible. In other words,

when studying the effect of pH of the solution upon the antigenic properties of a protein we are probably studying the relation of the pH to the properties of the protein and not to the properties of its cleavage products. Some evidence to prove that this is the case will be brought out later in this paper. The acid and alkaline solutions of protein used in these experiments have exceedingly low hydrogen and hydroxyl ion concentrations, are held at low temperatures and for short periods of time. It is, however, a distinct possibility—indeed a probability—that a certain amount (undoubtedly exceedingly minute) of hydrolysis and tautomerism occurs in the solute. Whether it is to reversible or to irreversible changes produced in the protein molecule to which we must ascribe the pH effects noted is beyond the scope of this work.

## EXPERIMENTS ON ACTIVE ANAPHYLAXIS

### *Gelatin*

In our first experiments we chose the anaphylactogenic properties of proteins for the study of pH effects, because the well known specificity of the reaction offered a means of measuring qualitative as well as quantitative changes. Our first studies were made with powdered gelatin. Although the anaphylactogenic properties of this protein have been questioned (Wells, 1920, 1921) we chose it because most of the physico-chemical work of Loeb was done with gelatin and because we considered it possible that the discrepancies in the literature concerning its antigenic potency might be due to differences in the acidity of gelatin solutions used by different investigators. We failed to produce anaphylaxis in guinea pigs with sensitizing and intoxicating doses of gelatin up to 0.05 gram introduced intravenously or intraperitoneally in solutions at its iso-electric point (pH = 4.7) and in more acid and in more alkaline solutions.

### *Ovalbumin*

Our next series of experiments on active anaphylaxis were conducted with crystallized hen ovalbumin prepared by the

method of Plimmer (1915). Preparations of the protein were recrystallized several times and were dialyzed against running tap water for two or three weeks and against repeated changes of distilled water during two or three days. Therefore we felt reasonably certain that we were working with an isolated protein of animal origin in a reasonably pure state. It is important to note that we took every precaution in the preparation of the protein and of its solutions to avoid even the barest possibilities of contamination with foreign proteins. All glassware (beakers, flasks, stirring rods, pipettes, etc.) used was either new or was carefully washed with soap and water, hot potassium dichromate-sulfuric acid cleaning fluid, tap water and distilled water and whenever possible soaked in the cleaning fluid for at least twenty-four hours before used. The distilled water used in these experiments was prepared by careful distillation in a Barnstead Still, was usually free from ammonia and had pH values in the range 5.6 to 6.3.

Ovalbumin solutions were prepared by weighing out the necessary quantity of protein and dissolving in the appropriate volume of distilled water. By this method we obtain what is customarily referred to as a neutral solution of the protein. As a matter of fact, if the concentration of protein is not very low (i.e., not less than about 5 mgm. per cubic centimeter), the solution is not *neutral,* but has the pH of $4.8 \pm 0.1$ and is really a solution of the protein at its isoelectric point. To prepare ovalbumin solutions at specific hydrogen-ion concentrations two methods have been used in this laboratory, both of which are simple and give results whose accuracy satisfied the requirements of these experiments. If the protein (in each of the concentrations which is to be utilized) is once titrated with acid and with alkali and the curve of pH against acid and alkali additions plotted (the so-called "titration curve") any desired pH may be obtained in a similar protein solution by interpolating on the graph and adding the appropriate quantity of acid or alkali to give the desired pH. The other method used was to add to 10 cc. portions of the ovalbumin solution in water the appropriate indicator of the Clark and Lubs series (in most of our work this

was thymol blue, acid and alkaline ranges) and to titrate to the desired pH with NaOH (N/20 or N/100) and HCl (N/5) solutions and then add the appropriate quantity of N/5 acid or alkali to a fraction of the original aqueous solution of protein in water.

In preliminary experiments we used the intravenous as well as intraperitoneal route for inoculation. Because the results obtained by the two methods were nowise significantly different and for reasons of manipulative convenience the intraperitoneal route was used in all the later experiments reported here. With a sensitizing dose of 0.005 gram, a sensitizing period of fourteen or more days and an intoxicating dose of 0.050 gram we obtained anaphylaxis in the guinea-pig uniformly. In our judgment of the signs of acute anaphylaxis we have followed Coca (1920). Of the seven criteria laid down by Wells (1921) we have followed all but the fourth (reaction of isolated virgin uterus strip) and fifth (amelioration of bronchial spasm with atropin and epinephrin).

In tables 1, 2 and 3 we have reproduced the data from our experiments 9, 10 and 11. We chose pH = 2.0 to 2.5 as sufficiently hyperacidic with respect to the iso-electric point of ovalbumin (pH = 4.8) to represent protein which—in the nomenclature of Loeb—was in the form of Ovalbumin$^{+}$, $Cl^{-}$; and pH = 9.0 to 10.0 as sufficiently hypo-acidic to represent protein in the form of $Na^{+}$, Ovalbumin$^{-}$. In all cases the sensitizing and intoxicating doses were contained in 1 cc. of solution. The reactions of the animals to the acid and alkali contained in the protein solutions were exceedingly mild as judged by the differences in the reactions of the various groups at the time of sensitization.

The data in tables 1, 2 and 3 indicate first that the sensitization produced in the guinea pig by the protein in solutions of pH 2.0 to 2.5, 4.8 and 9.0 to 10.0 is evidenced by anaphylactic reactions whose characteristics are not appreciably modified by the pH of the solution used for intoxication. Second, the data indicate clearly that the more acidic the sensitizing solution the more acute and the more often fatal the anaphylactic response, regardless of the pH of the intoxicating solution. This is evi-

TABLE 1

*Experiment 9. Active anaphylaxis in guinea-pigs*

Sensitizing dose: 0.005 gram of ovalbumin
Intoxicating dose: 0.50 gram of ovalbumin
Interval between sensitization and intoxication: sixteen days.
*Isoelectric point for ovalbumin: pH = 4.7*
Acid solution of ovalbumin, pH = 2.0
Unadjusted solution of ovalbumin, pH = 4.8
Alkaline solution of ovalbumin, pH = 10.0

| NUMBER | SENSITIZATION | INTOXICATION | RESULTS |
|---|---|---|---|
| | Protein in | Protein in | |
| 1 | Acid solution | Alkaline solution | Fatal, acute, anaphylaxis |
| 2* | Acid solution | Alkaline solution | Fatal, acute, anaphylaxis |
| 3 | Alkaline solution | Acid solution | Acute anaphylaxis |
| 4 | Alkaline solution | Acid solution | Acute anaphylaxis |
| 5 | Alkaline solution | Acid solution | Acute anaphylaxis |

* The third animal of this series was lost from a pneumonic infection during the sensitizing period of incubation.

TABLE 2

*Experiment 10. Active anaphylaxis in guinea-pigs*

Sensitizing dose: 0.005 gram of ovalbumin
Intoxicating dose: 0.05 gram of ovalbumin
Interval between sensitization and intoxication: fourteen days

| NUMBER | SENSITIZATION | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 2.0 | Alkaline solution | 10.0 | Fatal, acute, anaphylaxis |
| 2 | Acid solution | 2.0 | Alkaline solution | 10.0 | Fatal, acute, anaphylaxis |
| 3 | Acid solution | 2.0 | Alkaline solution | 10.0 | Acute anaphylaxis |
| 4 | Alkaline solution | 10.0 | Acid solution | 2.0 | Mild anaphylaxis |
| 5 | Alkaline solution | 10.0 | Acid solution | 2.0 | Mild anaphylaxis |
| 6 | Alkaline solution | 10.0 | Acid solution | 2.0 | Mild anaphylaxis |

*Acute anaphylaxis:* Includes both paralytic and respiratory reactions.
*Mild anaphylaxis:* Paralytic reaction with no or slight respiratory reaction.
Solutions incubated one hour after adjustment of pH and before inoculations.

TABLE 3

*Experiment 11. Active anaphylaxis in guinea-pigs*

Sensitizing dose: 0.005 gram of ovalbumin
Intoxicating dose: 0.05 gram of ovalbumin
Interval between sensitization and intoxication: sixteen days

| NUMBER | SENSITIZATION | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 2.5 | Alkaline solution | 9.0 | Acute anaphylaxis |
| 2 | Acid solution | 2.5 | Alkaline solution | 9.0 | Fatal, acute anaphylaxis |
| 3 | Acid solution | 2.5 | Unadjusted solution | 4.8 | Fatal, acute anaphylaxis |
| 4 | Acid solution | 2.5 | Unadjusted solution | 4.8 | Fatal, acute anaphylaxis |
| 5 | Acid solution | 2.5 | Acid solution | 2.5 | Fatal, acute anaphylaxis |
| 6 | Acid solution | 2.5 | Acid solution | 2.5 | Fatal, acute anaphylaxis |
| 7 | Unadjusted solution | 4.8 | Alkaline solution | 9.0 | Acute anaphylaxis |
| 8 | Unadjusted solution | 4.8 | Alkaline solution | 9.0 | Fatal, acute anaphylaxis |
| 9 | Unadjusted solution | 4.8 | Unadjusted solution | 4.8 | Mild anaphylaxis |
| 10 | Unadjusted solution | 4.8 | Acid solution | 2.5 | Mild anaphylaxis |
| 11 | Unadjusted solution | 4.8 | Acid solution | 2.5 | Mild anaphylaxis |
| 12 | Alkaline solution | 9.0 | Alkaline solution | 9.0 | Mild anaphylaxis |
| 13 | Alkaline solution | 9.0 | Unadjusted solution | 4.8 | Mild anaphylaxis |
| 14 | Alkaline solution | 9.0 | Unadjusted solution | 4.8 | No anaphylaxis |
| 15 | Alkaline solution | 9.0 | Acid solution | 2.5 | Mild anaphylaxis |
| 16 | Alkaline solution | 9.0 | Acid solution | 2.5 | Mild anaphylaxis |

*Acute anaphylaxis:* Paralytic and respiratory reactions.
*Mild anaphylaxis:* Paralytic with no or slight respiratory reaction.
Solutions incubated one hour at 20°C. after adjustment of pH and before inoculations.

dence of one kind which indicates either that the effect of the pH upon the protein is not to break it down into protein cleavage products of different sensitizing potency or specificity, or that

hydrolytic or tautomeric changes which might render the protein non-antigenic are not very extensive. In a few control experiments we have, from time to time, prepared our acidic sensitizing solution from protein which had first been held for an hour at the alkaline pH. The responses elicited in the guinea pigs were similar to those consistently obtained with the acidulated protein solutions. It therefore appeared to us probable that the milder sensitizing potency of ovalbumin in alkaline solution is not to be attributed to the irreversible denaturing of the protein by the OH-ions although such evidence is not conclusive. For controls, guinea pigs which received 1 cc. of water adjusted to 2.0 to 2.5, 4.8 and 9.0 to 10.0 were held along with the ovalbumin-sensitized pigs and received the same intoxicating doses of ovalbumin in unadjusted acidic and alkaline solutions. We never elicited an anaphylactic response in these controls nor in others which received the sensitizing doses of ovalbumin and were "intoxicated" with the solutions lacking the protein.

That the effect of the acid and alkali is upon the protein and not upon the animal was indicated by data of two sorts. We had found, empirically, that to obtain such consistent results as are indicated in tables 1, 2 and 3 it was essential to hold the protein solutions at room or ice-chest temperature for at least one-half hour. Apparently there is a time factor of considerable importance concerned in the process of converting the protein antigen from equilibrium with one pH to equilibrium with another. Also we found that guinea-pigs which received sensitizing inoculations of ovalbumin in unadjusted solutions (pH = 4.8) and fifteen to thirty minutes later inoculations of acid or alkali equivalent to the quantity contained in the acidic or alkaline protein solutions gave—after a suitable incubation period and inoculation with protein solutions—anaphylactic responses similar to those elicited in guinea-pigs sensitized with unadjusted solutions of the protein. We therefore consider it justifiable to hold that the increased anaphylactogenic potency of acidulated ovalbumin solutions which is evidenced in these experiments is attributable to the effect of hydrogen-ion concentration upon the antigen and not upon the animal. Evidence that it is not directly

attributable to change in solubility of antigen with a shift in pH will be discussed below.

The data presented above do not show any important modification of intoxicating potency with changes in pH. We have some evidence—too fragmentary to report here—that such modification does occur under certain conditions. This phase of the problem is being studied more carefully now, with particular reference to its possible applications in the practice of serum therapy.

In tables 2 and 3 we have indicated that in *acute anaphylaxis* (fatal or non-fatal) we have observed all the classical signs of the reaction. In the reactions which we have designated as *mild anaphylaxis,* there were no or only very slight signs of the respiratory reaction, the "air hunger,"—one of the outstanding characteristics of acute anaphylaxis. In these mild anaphylactic responses the paralytic reactions, especially of the animal's hind quarters, were entirely typical. It is not clear to us whether the difference between these two types of anaphylactic responses are merely quantitative or not. This problem is being studied in our laboratories.

## EXPERIMENTS ON PASSIVE ANAPHYLAXIS

### *Ovalbumin*

It is well known that the variations in antibody response which may occur in different animals which have been given equal doses of antigen may be considerable. In animals which have been actively hypersensitized it is held that these variations may be magnified by differences in cellular absorption of antibody in addition to differences in total antibody response. Therefore, upon the advice of Prof. G. H. Smith, we repeated our ovalbumin experiments by the method of passive anaphylaxis thus making it reasonably certain that each guinea-pig received the same quantity of antibody. We sensitized a series of rabbits to ovalbumin by intraperitoneal inoculations of 5 cc. of water containing 0.125 gram of the protein. For one group of rabbits the solution had been adjusted to pH = 2.5, for a second group to pH = 9.5 and for the third group was unadjusted (pH = 4.8). The solu-

tions were held in the ice chest for four hours after the adjustments of pH. Immediately before each set of inoculations, the pH of each ovalbumin solution was measured again. They were found to be of the stated values. All the usual precautions were taken to insure the chemical cleanliness as well as the sterility of glassware, syringes, etc. The batch or protein used in these experiments was the same as that used in the experiments reported in tables 1, 2 and 3. Twenty-two days after they received these sensitizing inoculations of protein the rabbits were bled from the marginal ear veins. The blood was caught in carefully cleaned sterile centrifuge tubes, held in the ice chest until clotting had occurred; the clots were broken with clean glass rods; the tubes were held in the ice chest over night and centrifugalized next morning. The sera were separated and were used directly for inoculation into guinea-pigs that had not been used before for any experimental purpose. As indicated in table 4, for the first three groups of guinea-pigs, three rabbit sera were used, 1 cc. of a particular serum being inoculated intraperitoneally into each of the three pigs in a group. A fourth group of three guinea-pigs (animals 10, 11 and 12) served as a control and each animal in this group received 1 cc. of serum from a rabbit that had never been given ovalbumin. These guinea-pigs showed no reactions to the serum inoculations. Twenty-four hours later all the guinea-pigs were given intraperitoneal inoculations of 0.05 gram of ovalbumin contained in 1 cc. of water (unadjusted, acidulated and alkalinized; pH = 4.8, 2.5 and 9.5, respectively). The results are shown in the last column of table 4. In table 5 is shown an entirely similar set of data, the guinea-pigs having been sensitized with sera from the same rabbits used in experiment 12, table 4. The only differences are that the sera for experiment 15, table 5 were obtained by bleeding the rabbits from the heart on the twenty-sixth day after sensitization and that 1.5 cc. (instead of 1 cc.) of serum was inoculated into each guinea-pig. The intoxicating doses were 0.05 gram of protein as in the previous experiments.

Although all the guinea-pigs survived experiments 12 and 15, the differences in the acuteness of the anaphylactic response were

clear. The indications are that under the conditions of these experiments the sera of rabbits sensitized with ovalbumin are more effective in passively sensitizing guinea-pigs the more acidic the sensitizing solution introduced into the rabbits, and that the pH of the intoxicating solutions does not affect the acuteness of

TABLE 4

*Experiment 12. Passive anaphylaxis in guinea-pigs*

Sensitization of rabbits: 0.125 gram ovalbumin

Sensitization of guinea-pigs: 1 cc. of rabbit serum twenty-two days after sensitization.

Intoxication of guinea-pigs: 0.05 gram ovalbumin.

| NUMBER | SENSITIZATION WITH SERA FROM RABBITS | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 2.5 | Acid solution | 2.5 | Acute anaphylaxis |
| 2 | Acid solution | 2.5 | Unadjusted solution | 4.8 | Acute anaphylaxis |
| 3 | Acid solution | 2.5 | Alkaline solution | 9.5 | Acute anaphylaxis |
| 4 | Unadjusted solution | 4.8 | Acid solution | 2.5 | Anaphylaxis |
| 5 | Unadjusted solution | 4.8 | Unadjusted solu- | 4.8 | Anaphylaxis |
| 6 | Unadjusted solution | 4.8 | Alkaline solution | 9.5 | Anaphylaxis |
| 7 | Alkaline solution | 9.5 | Acid solution | 2.5 | Mild anaphylaxis |
| 8 | Alkaline solution | 9.5 | Unadjusted solution | 4.8 | Mild anaphylaxis |
| 9 | Alkaline solution | 9.5 | Alkaline solution | 9.5 | Mild anaphylaxis |
| 10 | (No protein) | | Acid solution | 2.5 | No anaphylaxis |
| 11 | (No protein) | | Unadjusted solution | 4.8 | No anaphylaxis |
| 12 | (No protein) | | Alkaline solution | 9.5 | No anaphylaxis |

*Acute anaphylaxis:* Paralytic and respiratory reactions.
*Anaphylaxis:* Mild paralytic and respiratory reactions.
*Mild anaphylaxis:* Paralytic with no or slight respiratory reactions.
Solutions incubated four hours after adjustment of pH and before inoculations.

the specific response elicited in the passively sensitized guinea-pigs. Also, because of the continuity in the relative mildness of the reaction with increasing alkalinity of the sensitizing protein, it appears that the essential difference between the *acute anaphylaxis* and the *mild anaphylaxis* (absence or mildness of the "air hunger") are quantitative rather than qualitative.

### *Edestin*

In parallel with the experiments on passive anaphylaxis with ovalbumin, we conducted experiments with the hemp seed globulin, edestin. We chose this protein to confirm our findings upon

TABLE 5

*Experiment 15. Passive anaphylaxis in guinea-pigs*

Sensitization of rabbits: 0.125 gram ovalbumin

Sensitization of guinea-pigs: 1.5 cc. of rabbit serum twenty-six days after sensitization.

Intoxication of guinea-pigs: 0.05 gram ovalbumin.

| NUMBER | SENSITIZATION WITH SERA FROM RABBITS | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 2.5 | Acid solution | 2.5 | Acute anaphylaxis |
| 2 | Acid soution | 2.5 | Unadjusted solution | 4.8 | Acute anaphylaxis |
| 3 | Acid solution | 2.5 | Alkaline solution | 10.0 | Acute anaphylaxis |
| 4 | Unadjusted solution | 4.8 | Acid solution | 2.5 | Mild anaphylaxis |
| 5 | Unadjusted solution | 4.8 | Unadjusted solution | 4.8 | Mild anaphylaxis |
| 6 | Unadjusted solution | 4.8 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 7 | Alkaline solution | 10.0 | Acid solution | 2.5 | Mild anaphylaxis |
| 8 | Alkaline solution | 10.0 | Unadjusted solution | 4.8 | Mild anaphylaxis |
| 9 | Alkaline solution | 10.0 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 10 | No protein | | Acid solution | 2.5 | No anaphylaxis |
| 11 | No protein | | Unadjusted solution | 4.8 | No anaphylaxis |
| 12 | No protein | | Alkaline solution | 10.0 | No anaphylaxis |

*Acute anaphylaxis:* Paralytic and respiratory reactions.

*Mild anaphylaxis:* Paralytic with no or slight respiratory reaction.

Solutions incubated two hours at room temperature after adjustment of pH and before inoculations.

ovalbumin because like ovalbumin it is known to be an effective anaphylactogen, because it can be crystallized and obtained in reasonably pure state and because it differs from ovalbumin in being a globulin instead of an albumin, and is of plant instead of animal origin. Also, its iso-electric point is much closer to the neutral point of water than is that of ovalbumin. Its iso-electric

point is stated by Rona and Michaelis (1910) to be $1.3 \times 10^{-7}$, i.e., pH = 6.9, and by Hitchcock (1922) to lie between pH = 5.5 and pH = 6.0.

The edestin used in these experiments was prepared from hemp seed by the method which is based upon the fact that edestin is readily soluble in 5 per cent NaCl solution at 60°C. and is insoluble in this reagent at lower temperatures. Ground hemp seed was extracted at 60°C. for one-half hour with 5 per cent NaCl solution. The extract was strained through cheesecloth and filtered through paper. The filtrate was diluted with water as far as possible without precipitating the globulin (at 60°C.). The solution was then permitted to cool very slowly. The crystals of edestin separated out. They were filtered off after the solution had been cooled to 5°C., were washed with cold 3 per cent NaCl solution, resuspended in distilled water and dialysed against running tap water until free from NaCl. The crystals were then filtered off, washed with repeated changes of distilled water, then with alcohol and ether and were dried in a desiccator. The preparation was tested for its usual protein reactions and its solubilities and was considered to be a sufficiently pure preparation of edestin. Its iso-electric point appeared to be at about pH = 6.5.

For sensitizing the rabbits, a solution of edestin was prepared by saturating a 5 per cent NaCl solution at 60°C., filtering through paper, heating the filtrate to 61°C. and washing over the edestin residue on the filter paper three times. The edestin concentration was 0.007 gram per cubic centimeter. The pH of the solution was 6.0. Adjustments of pH to the values stated in tables 6 and 7 were made with HCl and NaOH by the second of the two methods described above for ovalbumin. The solutions were held in the ice chest after pH adjustment until the time of inoculations. The pH of each solution was read again immediately before the inoculations and was found in each case to have the stated value. The edestin solutions used for intoxicating the guinea-pigs were prepared by the same method. When an edestin solution of this kind cools below 60°C. it becomes turbid. This turbidity is converted into a compact, fibrous

precipitate when the solution is adjusted to pH 2.5 to 3.0 with HCl and dissolves when the solution is adjusted to 10.0 with NaOH. As stated in the tables, 2 cc. (0.014 gram) of the edestin solutions were used for sensitization of the rabbits. In experi-

TABLE 6

*Experiment 13. Passive anaphylaxis in guinea-pigs*

Sensitization of rabbits: 2 cc. of 5 per cent NaCl solution containing 0.014 gram edestin

Sensitization of guinea-pigs: 1 cc. of rabbit serum twenty-three days after sensitization

Intoxication of guinea-pigs: 1 cc. doses of the standard edestin solution (1 cc. = 0.007 gram edestin

| NUMBER | SENSITIZATION WITH SERA FROM RABBITS | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 3.0 | Acid solution | 3.0 | Mild anaphylaxis |
| 2 | Acid solution | 3.0 | Unadjusted solution | 6.2 | Mild anaphylaxis |
| 3 | Acid solution | 3.0 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 4 | Unadjusted solution | 6.2 | Acid solution | 3.0 | Anaphylaxis |
| 5 | Unadjusted solution | 6.2 | Unadjusted solution | 6.2 | No anaphylaxis |
| 6 | Unadjusted solution | 6.2 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 7 | Alkaline solution | 10.0 | Acid solution | 3.0 | No anaphylaxis |
| 8 | Alkaline solution | 10.0 | Unadjusted solution | 6.2 | No anaphylaxis |
| 9 | Alkaline solution | 10.0 | Alkaline solution | 10.0 | No anaphylaxis |
| 10 | (No protein) | | Acid solution | 3.0 | No anaphylaxis |
| 11 | (No protein) | | Unadjusted solution | 6.2 | No anaphylaxis |
| 12 | (No protein) | | Alkaline solution | 10.0 | No anaphylaxis |

*Anaphylaxis:* Mild paralytic and respiratory reactions

*Mild anaphylaxis:* Paralytic with no or slight respiratory reactions

Standard edestin solution made by saturating a 5 per cent NaCl solution with edestin at 60°C. Solutions incubated two hours after adjustment of pH and before inoculations.

ment 13 (table 6) the rabbits were bled from the marginal ear veins twenty-two days after sensitization and in experiment 14 (table 7) the same rabbits were bled from the heart twenty-six days after sensitization. In experiment 13, 1 cc. of each rabbit serum was inoculated intraperitoneally into each guinea-

pig and in experiment 14, 1.5 cc. The rabbit sera were prepared as described above in the ovalbumin experiments. The guinea-pigs were given the intoxicating doses of edestin twenty-four hours after they had received the passively sensitizing rabbit

TABLE 7

*Experiment 14. Passive anaphylaxis in guinea-pigs*

Sensitization of rabbits: 2 cc. of 5 per cent NaCl solution containing 0.014 gram edestin

Sensitization of guinea-pigs: 1.5 cc. of rabbit serum twenty-seven days after sensitization.

Intoxication of guinea-pigs: 1.5 cc. doses of the standard edestin solution (1.5 cc. = 0.011 gram).

| NUMBER | SENSITIZATION WITH SERA FROM RABBITS | | INTOXICATION | | RESULTS |
|---|---|---|---|---|---|
| | Protein in: | pH | Protein in: | pH | |
| 1 | Acid solution | 2.5 | Acid solution | 2.5 | Acute anaphylaxis |
| 2 | Acid solution | 2.5 | Unadjusted solution | 6.2 | Acute anaphylaxis |
| 3 | Acid solution | 2.5 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 4 | Unadjusted solution | 6.2 | Acid solution | 2.5 | Anaphylaxis |
| 5 | Unadjusted solution | 6.2 | Unadjusted solution | 6.2 | Anaphylaxis |
| 6 | Unadjusted solution | 6.2 | Alkaline solution | 10.0 | Anaphylaxis |
| 7 | Alkaline solution | 10.0 | Acid solution | 2.5 | Anaphylaxis |
| 8 | Alkaline solution | 10.0 | Unadjusted solution | 6.2 | Anaphylaxis |
| 9 | Alkaline solution | 10.0 | Alkaline solution | 10.0 | Mild anaphylaxis |
| 10 | (No protein) | | Acid solution | 2.5 | No anaphylaxis |
| 11 | (No protein) | | Unadjusted solution | 6.2 | No anaphylaxis |
| 12 | (No protein) | | Alkaline solution | 10.0 | No anaphylaxis |

*Acute Anaphylaxis:* Paralytic and respiratory reactions.

*Anaphylaxis:* Mild paralytic and respiratory reactions.

*Mild Anaphylaxis:* Paralytic with no or slight respiratory reactions.

Standard edestin solution made by saturating a 5 per cent NaCl solution with edestin at 60°C. Solutions incubated two and one-half hours after adjustment of pH and before inoculations.

sera. The guinea-pigs used in experiment 14 (table 7) were animals which had survived the ovalbumin experiment tabulated in table 4. It was tacitly assumed that the specificity of the anaphylaxis reaction permitted the use in an edestin experiment

of animals which had been used previously in an ovalbumin experiment. All the pigs used in these experiments weighed approximately 300 grams. The last columns of tables 6 and 7 indicate the anaphylactic reactions of the guinea-pigs.

It is evident that the reactions in these experiments were not as severe as in the ovalbumin experiments. Here, as in the ovalbumin experiments, the reactions are more acute the more acid the protein used to sensitize the rabbits. The pH of the intoxicating protein appeared to play a more important rôle than in the ovalbumin experiments. It will be noted both in table 6 and in table 7 that whenever there is an appreciable variation in the severity of the reaction in any one group of guinea-pigs, the reaction is less severe the more alkaline the intoxicating dose. This observation is entirely in harmony with findings in experiments not reported here, that in the alkaline solutions the proteins are less potent in intoxicating the sensitized guinea-pigs. The variation in the opposite direction in experiment 11, animals 7 to 11 (table 3) is the only exception that we have encountered.

## EXPERIMENTS ON PRECIPITINS

### *Ovalbumin*

It is the tendency in immunological literature to stress the parallelism between the occurrence of anaphylactic and precipitating antibodies in immune sera. Indeed some workers incline to the view that the two are identical (vide the review by Coca, 1920). We have therefore conducted some titrations of the precipitins in the sera of the rabbits which had received intraperitoneal inoculations of ovalbumin in water (pH = 4.8) and in aqueous adjusted solutions, pH 2.0 to 2.5 and 9.0 to 10.0. We started the experiment with groups of three animals for each form of sensitizing protein. Several of the animals died of intercurrent infections during the course of the incubation interval. The technic and the materials were the same as those described above in the anaphylaxis experiments. The solutions were kept in the ice chest four hours after adjustment of pH and before inoculations. The pH reading of each solution was checked immediately before it was inoculated into the animals.

The ovalbumin antigen solution used in making the test for precipitating antibodies was prepared by dissolving 1 gram of ovalbumin in 100 cc. of 0.8 per cent NaCl solution. This solution had a pH = 4.8. In the first three titrations which were made it was diluted with 0.8 per cent NaCl. When added to the sera the mixtures showed pH values 6.8 to 7.2. In a later series of titrations a 10 cc. portion was adjusted to pH = 7.0 with N/5 NaOH and the solution was filtered clear through paper. Dilutions of antigen were made from this solution with

TABLE 8

*Titrations of precipitins in rabbit sera*

Sensitization with 5 cc. of ovalbumin solution containing 0.125 gram protein.
Animals bled thirty days after sensitization.
Acid solution of ovalbumin pH = 2.5.
Unadjusted solution of ovalbumin pH = 4.8.
Alkaline solution of ovalbumin pH = 9.5.

| SERA | | OVALBUMIN IN 0.8 PER CENT NaCl, 1 PART IN | | | | | |
|---|---|---|---|---|---|---|---|
| NUMBER | Sensitization with protein in: | 100 | 1,000 | 10,000 | 100,000 | 1,000,000 | 10,000,000 |
| 11 | Acid solution | + | + | ± | − | − | − |
| 13 | Acid solution | + | + | + | − | − | − |
| 27 | Acid solution | ++ | ++ | ++ | ± | + | − |
| 22 | Unadjusted solution | − | − | − | − | − | − |
| 30 | Unadjusted solution | ± | − | − | − | − | − |
| 20 | Alkaline solution | − | − | − | − | − | − |
| 25 | Alkaline solution | + | − | − | − | − | − |

++, heavy ring of precipitate; +, definite ring of precipitate; ±, doubtful ring of precipitate; −, no ring of precipitate.

an 0.8 per cent NaCl solution of pH = 7.0 to give albumin concentrations of one part in $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$. The pH of each dilution was read and found to be 7.0 ± 0.1. The precipitin tests were made in tubes prepared from glass tubing of 5 mm. bore. One-tenth cubic centimeter of undiluted serum was overlaid in each case by 0.1 cc. of the diluted antigen. The formation of a ring of precipitated material at the interphase of the two components was observed for the two hours succeeding the addition of antigen to serum. Control tests were made with sera and saline and with saline and antigen combinations.

No spontaneous ring precipitates were observed. In table 8 we have arranged the results with sera obtained from the rabbits thirty days after sensitization. They represent one of three sets of concordant titrations.

It is evident from the data of table 8 that the precipitating antibodies were present in much greater concentration in the sera of those rabbits which had been sensitized with acidulated ovalbumin solution than in the sera from the rabbits which had received the protein in unadjusted or in alkaline solution. These results were confirmed by another set of titrations upon sera from the same animals obtained forty-six days after sensitization, although all the titers had fallen. Sera 27, 30 and 20 had all been used for the passive sensitization of the guinea-pigs of experiments 12 and 15 (tables 4 and 5).

*Edestin*

The titrations of precipitins in rabbits which had been sensitized with edestin were conducted in the same manner as with ovalbumin. The rabbits were sensitized in the same manner and with the same quantities of protein used in sensitizing the rabbits for the edestin passive anaphylaxis experiments. The antigen was prepared by making the same type of edestin solution in 5 per cent NaCl (described above) and diluting this with 0.8 per cent NaCl to give approximately 1 part of protein in 1000, 10,000, 100,000, 1,000,000 and 10,000,000.[3] Even in the 1:1000 dilution there was not sufficient globulin precipitated to interfere with the test. The technique of the test was the same as that described for ovalbumin. The results of the first titration, made with sera obtained thirty days after sensitization, are summarized in table 9.

The results of the experiment summarized in table 9 are very puzzling. They appear to indicate that the more alkaline sensitizing doses of edestin were more effective precipitinogens than the acidulated doses. Sera 21, 10 and 29 had been utilized

[3] Determinations of edestin in solution showed that the dilution was 30 per cent higher in each case than the indicated value.

for the passive sensitization of the guinea-pigs of experiments 13 and 14 (tables 6 and 7). From those experiments it had appeared that the acidulated solution of edestin was the most potent in arousing in the rabbits the production of anaphylactic antibody. The failure of the precipitin titers to parallel the anaphylaxis titers was a very surprising finding in the light of the many contributions in the literature and of our ovalbumin experiments which indicated this parallelism. We therefore undertook to repeat these titrations.

TABLE 9

*Titrations of precipitins in rabbit sera*

Sensitization with 2 cc. of edestin solution containing 0.014 gram protein in 5 per cent NaCl.

Animals bled thirty days after sensitization.

Acid solution of edestin pH = 3.0

Neutral solution of edestin pH = 6.9

Alkaline solution of edestin pH = 10.0

| SERA | | EDESTIN IN 0.8 PER CENT NaCl, 1 PART IN | | | | |
|---|---|---|---|---|---|---|
| Number | Sensitization with protein in: | 1,000 | 10,000 | 100,000 | 1,000,000 | 10,000,000 |
| 12 | Acid solution | − | − | − | − | − |
| 21 | Acid solution | − | − | − | − | − |
| 10 | Neutral solution | + | + | − | − | − |
| 17 | Neutral solution | * | * | * | * | * |
| 8 | Alkaline solution | + | + | − | − | − |
| 28 | Alkaline solution | + | + | − | − | − |
| 29 | Alkaline solution | + | − | − | − | − |

* Test doubtful. Serum turbid.

+, definite ring of precipitate; −, no ring of precipitate.

The edestin-sensitized rabbits were again bled from the heart, this time forty-six days after sensitization. Two days later the sera were titrated for precipitins. The antigen used in these tests was prepared by a somewhat different method. To avoid the use of 5 per cent NaCl solution to dissolve the edestin, we made use of the fact that this globulin dissolves more readily in alkaline than in neutral or acid solutions. White and Avery (1913) have also had recourse to this device. In their experiments with edestin they used solutions of the protein in $Na_2CO_3$

solution.[4] We used solutions of edestin prepared by adding 0.1 gram to 100 cc. of 0.8 per cent NaCl solution (pH = 5.5; considerable portion of the protein undissolved). To 10 cc. of this solution N/20 NaOH was added (3 drops) until the solution was clear (pH = circa 10.2). From this alkaline solution (1 part protein in 1000 of saline) further dilutions were made with 0.8 per cent NaCl solution which had been adjusted to pH = 10.0 so that the final concentrations of protein in alkaline 0.8 per cent

TABLE 10

*Titrations of precipitins in rabbit sera*

Sensitization with 2 cc. of edestin solution containing 0.014 gram protein in 5 per cent NaCl.

Animals bled forty-six days after sensitization.

Acid solution of edestin pH = 3.0

Neutral solution of edestin pH = 6.9

Alkaline solution of edestin pH = 10.0

| SERA | | EDESTIN IN 0.8 PER CENT NaCl, pH = 10.2, 1 PART IN | | | | |
|---|---|---|---|---|---|---|
| Number | Sensitization with protein in: | 1,000 | 10,000 | 100,000 | 1,000,000 | 10,000,000 |
| 12 | Acid solution | ± | − | − | − | − |
| 21 | Acid solution | ± | − | − | − | − |
| 23 | Acid solution | ± | − | − | − | − |
| 7 | Neutral solution | ± | − | − | − | − |
| 10* | Neutral solution | + | + | ± | − | − |
| 17* | Neutral solution | + | + | ± | − | − |
| 8 | Alkaline solution | ± | − | − | − | − |
| 28 | Alkaline solution | + | + | − | − | − |
| 29 | Alkaline solution | ± | − | − | − | − |

* Due to the loss of tags from rabbits, we do not know which of these is no. 10 and which no. 17.

+, definite ring of precipitate; ±, slight ring of precipitate; −, no ring of precipitate.

NaCl were 1 part in $10^4$ to 1 part in $10^7$ by decimal dilutions. The results of the test are shown in table 10.

White and Avery (1913) have pointed out that in alkaline ($Na_2CO_3$) solutions of edestin more concentrated than 1:1000 spontaneous precipitation occurs upon standing or upon contact

[4] We wish to express our thanks to Dr. White for having called our attention to this procedure and to the results described in their paper.

with diluted (1:10) normal serum. This observation may account for the ± reaction observed with all our sera overlaid with antigen 1:1000.

The data of table 10 confirm those of table 9 in the demonstration that the precipitin titer was higher in the sera of rabbits sensitized with neutral or alkaline solutions of edestin than in the sera of rabbits sensitized with the acid solution of the protein.

## DISCUSSION

We have observed that the guinea-pigs or rabbits which received acidic or alkaline solutions of protein showed no more discomfort or reaction after the inoculation than the animals which received unadjusted solutions. We are therefore led to the conclusion that the acid or alkali injected with the proteins in these experiments is not of physiological significance.

The enormous power of mammalian tissues and body fluids to maintain their neutrality is too well known to demand discussion here. It is the more surprising therefore—if the effects noted in these experiments are due to reversible changes in the antigen—that the pH of the solution in which a protein is suspended affects its antigenic properties. *A priori* it would be expected that the acid or alkali of the inoculated material would be very rapidly neutralized in the blood or in the peritoneal cavity and the protein converted into that state in which it exists at equilibrium with the hydrogen-ion concentration of the blood. From the data presented here it appears that this may not occur under the conditions of these experiments. It appears probable that although the fluids of the body may change the pH of the inoculated material very rapidly, the effect of the pH to which the protein has previously been exposed persists for a period of time sufficiently long after introduction into the animal body to allow the specific antigenic potencies of the protein to become evidenced by later specific antibody responses.

That the effect of pH is upon the protein and not upon the animal has been indicated by the data discussed on page 246. It is therefore justifiable to conclude that the observations reported in this paper are not concerned with the phenomenon of

"defence rupture" or "kataphylaxis" (vide Bullock and Cramer, 1919, Shearer, 1919–1920, Cramer and Gye, 1919–1920).

Until the results of further experiments become available it will not be possible to state definitely whether the effect of pH upon antigenic properties is qualitative or only quantitative. As we have already pointed out above, the indications are in favor of the latter assumption. From the experiments with ovalbumin it appears that sensitization with the protein at one pH is evidenced equally as well by intoxication with the protein in solutions of any of the pH values tested. The experiments with edestin show much the same results except that with the more alkaline intoxicating solutions the responses are commonly less acute regardless of the pH of the sensitizing antigen. When we have obtained the reactions termed "acute anaphylaxis" and "mild anaphylaxis" the gradient between them appears to have been continuous with change in pH of the sensitizing protein. We therefore incline to the view that the effect of pH upon anaphylactic antigen, under the conditions of these experiments, to modify its potency in arousing an antibody response is more marked than in modifying its specific intoxicating properties.

It had been tacitly assumed, when these experiments were begun, that within the range of pH 2.0 to 10.0 the proteins ovalbumin and edestin would be modified only in the physico-chemical sense and that they would remain unchanged in the ordinary chemical sense. We must, however, examine this phase of the problem more closely.

It is known that treatment with concentrated alkali may result in racemization of proteins (Kossel and Weiss, 1912; Dakin, 1912; Dakin and Dudley, 1913, 1913a; etc.) and that concurrently with a change in the optical activity a protein may be rendered non-antigenic (TenBroeck, 1914; Schmidt, 1917) and refractory to the proteolytic activities of pepsin, trypsin and erepsin *in vitro*, and non-digestible *in vivo* (Dakin and Dudley, 1913a). It is even highly probable that treatment with strong alkali produces changes in the protein molecule more fundamental than were suspected by Dakin. Inasmuch as we have found that the more acidic solutions of ovalbumin and of edestin are

more effective sensitizing antigens than the corresponding alkaline solutions, it might appear—at first sight—as though the effects of pH which we have observed can be attributed to the loss in antigenic potency which parallels racemization in alkaline solution. However, it is far from probable-although possible—that such an explanation is tenable. It is significant to recall that when proteins are racemized and lose their antigenic properties—as in the experiments of TenBroeck and of Schmidt—they are treated with concentrated alkali (N/2–N/3.5 NaOH). In the experiments reported here the alkaline solutions were never at pH greater than 10.2. Although an hydroxyl-ion concentration equivalent to pH 10.0 appears very high in a *physiological* sense, *chemically* it represents a very weakly alkaline solution (approximately N/10,000 NaOH). The optical properties of ovalbumin and edestin probably are not appreciably affected at room or ice-chest temperature at this alkalinity in the incubation periods used in these experiments, and hydrolytic and tautomeric changes probably could not be demonstrated with the methods available.

So far as we are aware, an activation of proteins by very dilute acids—the converse of inactivation (racemization) by concentrated alkalies—has never been demonstrated. It is interesting to recall that in our experiments acidulation of the antigens to pH 2.0–3.0 (approximately N/100–N/1000 solutions of HCl) resulted in increased anaphylactogenic potency as compared to the potency of antigens in unadjusted (slightly acid) solutions. This observation apparently has no counterpart in those phenomena which are associated with racemization.

In the experiments of Schmidt (1917) on "racemized" casein the loss of antigenic characteristics was determined by the method of complement fixation. In the experiments of TenBroeck (1914) the loss of antigenic power upon racemization of egg albumin was demonstrated by the complement fixation, specific precipitation and anaphylactic reactions. In our experiments with ovalbumin, precipitinogenic and anaphylactogenic properties were alike augmented by acid and diminished by alkali. Our experiments with edestin, however, gave a distinctly different

result. The same antigen which produced the highest anaphylactic produced the lowest precipitin result. The difficulties of technique involved in making precipitin titrations against a globulin must not be minimized. But if, in spite of the methodological difficulties, our edestin precipitin titers are accurate, it would appear that either the pH effects which we have observed are entirely distinct from those which occurr under the conditions of racemization or that under those conditions some proteins (i.e., edestin)—unlike egg elbumin and casein—may show an increase instead of a decrease in the potency of a particular antigenic (precipitinogenic) property. We incline to doubt that such would be the case and to adhere to our original hypothesis that the effects of pH manifest in our experiments are reversible and are associated with physico-chemical changes in the proteins which are more closely related to ionization than to hydrolytic or tautomeric changes. Modifications of these latter types, although they might account for the diminished antigenic potency of alkaline solutions of antigen, do not account for the augmented potency of acidulated as compared to unadjusted antigen solutions.

It would have been highly desirable—had it been feasible—to relate the results of our experiments to the influence of pH upon the solubilities of proteins. However, sufficient data is not available in the literature or in our laboratory. We have conducted some experiments upon the solubilities of ovalbumin and edestin and have carried them far enough to discover that the variations produced by such changes in pH as have been utilized in the anaphylaxis experiments do not parallel the pH effects upon antigenic potency.

In the first section of this paper we indicated that we were led into these studies by acquaintance with certain recent contributions upon the relation of the physico-chemical properties of protein solutions to the iso-electric point. The iso-electric points of the proteins which have been studied in these experiments have been mentioned repeatedly. The data reported here indicate that the antigenic properties of proteins bear certain significant relations to the iso-electric points. We incline to the view

that in studying the effect of pH upon antigenic properties the iso-electric point should be determined, if possible, and pH values related to the iso-electric value. Indeed the iso-electric point cannot be neglected in studies upon pure proteins in unbuffered or only slightly buffered media because of the tendency of proteins to maintain in their menstrua the pH at which they are iso-electric.[5]

It would have been of considerable interest—had it been possible—to obtain the precipitin titers of our sera at the same hydrogen-ion concentrations as were used for sensitizing the animals. Such proposed protocols were abandoned when it was found necessary to use alkaline solutions to dissolve the edestin and when it was found that for the precipitin test with ovalbumin the limiting pH values (4.5 to 9.5) observed by Mason (1922) were sound.

The studies reported in this first paper are being continued. Some applications of these findings to the properties of bacterial antigens and to the detoxication of sera used in the practice of serum therapy are being studied at present. We have been convinced by the experiments reported here that much of the confusion in the literature of anaphylaxis may be attributable to the failure in the past to measure or control the hydrogen-ion concentration of antigen solutions. It appears probable that the effects of the hydrogen-ion concentration upon the characteristics of antigens are as fundamental as their influences upon the metabolic phenomena of protoplasm.

## CONCLUSIONS

1. In our experiments, variations of pH did not affect the non-anaphylactogenic nature of gelatin.

2. Unadjusted solutions of crystallized hen ovalbumin in water were at pH = 4.8. When this solution was brought to pH = 2.0 to 2.5 the anaphylactogenic potency of the protein

[5] For a discussion of some of the physico-chemical phases of this phenomenon vide the papers by Eckweiler, Noyes and Falk (1921), Cohn (1922), and Hitchcock (1922).

was markedly increased. When it was brought to pH = 9.0 to 10.0 this antigenic potency was slightly decreased.

3. Unadjusted solutions of crystallized edestin had pH values of 6.0 to 6.9. The anaphylactogenic potency of this globulin was increased when the solution was adjusted to pH = 2.5 and was decreased when the solution was adjusted to pH = 10.0.

4. With changes in pH, the precipitinogenic potency of ovalbumin paralleled the anaphylactogenic potency.

5. With changes in pH, the precipitinogenic potency of edestin was decreased when the anaphylactogenic potency was increased, and was but slightly reduced when anaphylactogenic potency was decreased.

6. The results with edestin were obtained by passive sensitization of guinea-pigs with the sera of actively sensitized rabbits. The experiments with ovalbumin were first conducted by the method of active anaphylaxis in the guinea-pig and were confirmed by experiments upon guinea-pigs passively sensitized with the sera of actively sensitized rabbits.

7. The acuteness of the anaphylactic response elicited in the actively or passively sensitized guinea-pig varied significantly with the pH of the sensitizing solution of protein.

8. The influence of the pH of the intoxicating solution upon the acuteness of the anaphylactic response was more marked in the edestin than in the ovalbumin experiments.

9. The pH of an antigen solution affects antibody response through its influence upon the protein and probably not through its influences upon the animal.

10. When studying antigenic properties of substances, the influence of pH cannot be neglected unless it is first demonstrated to be negligible.

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# COMPLEMENT-DEFICIENT GUINEA-PIG SERUM

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A stock of guinea-pigs whose serum is naturally deficient in complement was reported from the Vermont State Agricultural Station in 1919 by H. D. Moore (1). The stock is of more than ordinary interest to the serologist since it furnishes a workable character in the blood of a common laboratory animal. Short articles have already appeared by Coca (2) and Ecker (3) on stock obtained from the same source.

Moore observed that 1 cc. of the fresh serum from this breed of guinea-pigs would not hemolyze a unit of sensitized blood corpuscles of the horse. The serum hemolyzed sensitized sheep and human corpuscles only when very large doses were used. The guinea-pigs were found to respond to immunization with bacteria as well as normal stocks do. The opsonic action of the serum was low. The complement-deficient guinea-pigs were less resistant to infection with *B. cholerae suis* than normal animals. The complement-deficient condition was found to be heritable. Coca observed that the serum contained the end-piece and mid-piece of complement but the so-called third-piece was lacking. He also found that the serum could be activated by the addition of a small amount of inactivated human or guinea-pig serum. Ecker found that the serum would hemolyze supersensitized corpuscles. This author corroborated Coca's observation concerning the activation of the deficient serum with human and guinea-pig serum and further observed that dog, horse, sheep and rabbit serum were very poor "activators." In a personal communication, Dr. F. A. Rich of the Vermont Agricultural Station has informed us that he and Downing found the complement-deficient condition to be a Mendelian character.

Dr. Rich very kindly supplied us with a number of the complement-deficient guinea-pigs. We have had the stock under observation for more than two years and have made a study of a number of different points.

## EXPERIMENTAL

### *The complement content of the deficient serum*

The serum of the complement-deficient guinea-pigs was tested for its capacity to lyze sensitized erythrocytes of the sheep, the ox and the domestic fowl. The reactions with sheep corpuscles will serve to illustrate our results. Washed sheep corpuscles were mixed with two units of specific hemolytic serum (rabbit serum) and then distributed in unit quantites in two sets of tubes. Varying quantites of normal guinea-pig serum, properly diluted, were added to one set of tubes and to the other set, corresponding quantites of serum from the deficient guinea-pigs. The serum from normal guinea-pigs in quantities of 0.01 cc. gave complete hemolysis while 0.8 cc. of serum from the deficient guinea-pigs gave only partial hemolysis. It is seen, therefore, that the serum from the deficient guinea-pigs is almost devoid of the power to lyse sensitized corpuscles of the sheep. Similar results were obtained with the corpuscles of the other species.

### *Activation of the deficient serum*

Coca found that the addition of small amounts of inactivated (56°C. for thirty minutes) human or guinea-pig serum to the deficient serum rendered the latter one-third as potent as the serum of normal guinea-pigs. We have studied a number of sera, both in the fresh state and after inactivation, with respect to their activating effects on the deficient serum. The lytic actions of the various mixtures of serum were tested on red blood cells in combination with 2 units of specific hemolytic serum.

Fresh human serum was found to be a very potent activator for the complement-deficient serum. We have tested certain human sera of which 0.5 per cent by volume added to the deficient serum rendered the latter as potent as serum from normal guinea-

pigs. These tests were made as follows: The deficient serum was diluted and measured into a series of serological tubes in such a way that each tube contained 0.02 cc. of serum. The fresh human serum, after proper dilution, was so added to the tubes that the amounts of serum in the series varied from 0.1 cc. to 0.00005 cc. The tubes were shaken and allowed to stand at room temperature for ten minutes. Two units of hemolytic serum were now added to each tube and then 1 unit of sheep corpuscles was added. Hemolysis occurrred in all the tubes which contained 0.0001 cc. or more of the fresh human serum, while there was no hemolysis, or only slight hemolysis, in the tubes that contained less than 0.0001 cc. of human serum. The hemolytic serum used in this experiment had been inactivated by heat and contained 10,000 units per cubic centimeter.

Since 0.0001 cc. of the human serum rendered 0.02 cc. of the deficient guinea-pig serum as potent as an equal amount of normal guinea-pig serum, it may be said that the human serum contains 10,000 units of "activator" per cubic centimeter. As a rule, however, fresh human serum contains only about 5000 units of "activator" per cubic centimeter, 1 per cent by volume usually being required to activate completely the deficient serum. Fresh guinea-pig serum tested in the same way has proved almost as potent an "activator" as human serum, 1 per cent by volume always giving complete activation. The complement-deficient serum is activated also by small amounts of dog, rabbit and cat serum, but it is not activated by the fresh serum of the ox, pig, duck, chicken, pigeon, sheep, frog, goat or mouse. It is interesting to note that frog serum complements almost as well as guinea-pig serum and yet frog serum fails to supply the missing component to the deficient serum.

Heating at 56°C. for thirty minutes does not materially reduce the activating power of human serum. Heated guinea-pig serum will also activate the deficient serum but its activating power is so reduced that it is necessary to add 5 per cent of heated serum instead of 1 per cent as was the case with the fresh serum, in order to activate completely the deficient serum. Heating at 56°C. for thirty minutes entirely destroys the activating power of rabbit, cat and dog sera.

It is seen that of the 14 sera tested only 5 possess the property of activating the deficient serum. These 5 sera vary among themselves with respect to heat; 56°C. for thirty minutes entirely destroys this property of the rabbit, cat and dog sera, materially reduces it in guinea-pig serum, but does not appreciably affect human serum.

The quantitative results obtained with the heated human and guinea-pig sera are dependent upon the manner in which the tests are performed. It will be recalled that the hemolytic serum was added to the complement mixture and not to the suspension of corpuscles. This procedure was adopted because it had been observed that when the corpuscles are previously sensitized the reactions were delayed and different end-results were obtained.

It may be of interest to point out here that the complement deficient serum could not be distinguished from normal guinea-pig serum in an anti-sheep human hemolytic system, if the titer of the immune serum were not greater than 1 to 5000. This deduction results from the fact that the immune human serum, even after inactivation, would contain 5000 units of "activator" in each cubic centimeter. The deficient serum would also function in a similar way in an anti-sheep guinea-pig hemolytic system, provided that the immune serum were not inactivated before use.

### *Activation of the deficient serum in vivo*

Complement-deficient guinea-pigs were given injections of human and normal guinea-pig sera and bled at varying intervals of time thereafter. The serum from the different specimens of blood was collected and tested for its complementing action. It was found that an intravenous injection of 2 cc. of fresh serum from normal guinea-pigs rendered the serum of the complement-deficient guinea-pigs as potent as that of normal animals. Three days after the injections were made, the serum of the treated guinea-pigs was even less potent than it had been before the normal serum was injected. In other experiments, normal serum was injected either intraperitoneally or subcutaneously. Here the

activation was not instantaneous but occurred from four to eighteen hours after the injections were made, apparently as the serum reached the circulation. The sera of these animals also returned to the previous non-complementing state within from three to four days. Practically the same results were obtained with human serum.

The most interesting point in these experiments is the short duration of the conferred activity. In the light of experience with various immune sera, one would not be led to believe that the bulk of the injected serum was eliminated within three days' time, particularly where homologous serum was used. It is possible that the tissues of the complement-deficient guinea-pigs are, in some way, antagonistic to the activating substance, which is in reality foreign to these animals. It is pointed out that activation in vivo involves a condition somewhat different from that in the test tube, since the serum from the in vivo activation is obtained after the blood has passed through the clotting stage. This would indicate that the third component is not materially affected as a result of the process of coagulation.

*Complement-deficient serum and supersensitized corpuscles*

Ecker reports that the complement-deficient serum hemolyses corpuscles which have been sensitized with 500 units of specific amboceptor. The susceptibility of the corpuscles to the lytic action of the deficient serum was attributed to the supersensitization per se, as is shown by the following statement:

> These observations indicate that a greatly increased amboceptor content is capable of rendering the sheep cells so sensitive that the complement-deficient serum induced lysis in doses which, in themselves, are entirely inactive in the presence of the usual amboceptor amounts.

Before Ecker's report appeared, we had observed that the deficient serum functioned as normal serum in hemolytic reactions in which large quantities of amboceptor were used. For example, an anti-sheep hemolytic serum (rabbit serum) which

gave a titer of 1:10,000[1] with 0.02 cc. of normal guinea-pig complement, gave a titer of 1:50 with 0.02 cc. of the deficient serum. The deficient serum required, therefore, 0.02 cc. or 200 units of hemolytic serum to produce 1 unit of hemolysis. The hemolytic serum had been recently collected and was inactivated by heat, 56°C. for thirty minutes. This precaution was observed because old sera will not sensitize corpuscles for minimal doses of the complement-deficient serum.

Since inactivated normal rabbit serum will not activate the complement-deficient serum, the hemolysis would seem to be due directly to the large number of hemolytic units themselves, as Ecker contends. Further investigation has shown this not to be the case and indicates that the complement-deficient serum was able to effect hemolysis in the presence of excessive amounts of amboceptor because the missing factor was supplied by the hemolytic serum, rather than because of the hypersensitiveness of the supersensitized cells. The evidence which we have accumulated on this point is recorded in the following observations:

*a. Observations with yeast adsorption.* It is a well known fact that treatment with yeast will remove the complementing property of fresh sera. The yeast adsorbs the so-called third or thermostable component of complement. Since the deficient serum lacks this same factor, it follows that normal guinea-pig serum, after it has been adsorbed with yeast, will not activate the deficient serum. On the other hand, treatment with yeast does not affect the titer of a hemolytic serum; that is, hemolysins are not adsorbed by yeast. If our contention is true that the deficient serum is able to hemolyze the supersensitized corpuscles because the large amount of immune serum used supplies the missing complement factor, it follows that no hemolysis should occur if the hemolytic serum is previously treated with yeast. The following experiment shows this to be the case: Fresh

[1] Washed sheep corpuscles diluted 1:4 in terms of whole blood were used in doses of 0.1 cc. (This quantity is equivalent to one-quarter of the standard unit of corpuscles.—Editor). The reagents were added to small serological tubes, made up to a total volume of 1 cc. Readings were made after one hour's incubation at 37°C.

guinea-pig serum was mixed with large amounts of powdered yeast (Fleischman's) and kept at 37°C for two hours. The yeast was removed from the serum by centrifugalization. This serum failed to activate the complement-deficient serum and would hemolyze sensitized corpuscles only when excessive amounts of hemolytic serum were used. In other words, this serum now had the same properties as the complement-deficient serum. A very potent hemolytic serum was also adsorbed with yeast. The adsorbed serum was titrated with both normal and complement-deficient guinea-pig serum as complement. The titer was found to be unchanged with normal serum as complement, but any number of units did not sensitize the corpuscles for the complement-deficient serum or for normal guinea-pig serum that had been adsorbed with yeast.

This observation seems to admit of but one conclusion, namely, that the supersensitized corpuscles are susceptible to hemolysis by the complement-deficient serum because the hemolytic serum supplies the missing factor and not because of the supersensitization per se; for, if sensitization were the essential thing, any immune serum which contained the requisite number of sensitizing units should render the corpuscles susceptible to lysis by the deficient serum.

*b. Experiments with hemolytic serum heated above 56°C.* It had been demonstrated by Hyde (5a) that the titer of a potent hemolytic serum is only slightly reduced by being heated at 60°C. for thirty minutes and that 66°C. for thirty minutes destroys only about three-fourths of its hemolytic power. In view of these facts, it became of interest to see whether hemolytic serum which had been heated above 66°C. would sensitize corpuscles for the complement-deficient serum.

The hemolytic serum that was used in this experiment had a titer of 1:10,000 after inactivation at 56°C. After being heated at 60°C. for thirty minutes, the titer was only slightly reduced, normal serum being used as complement, but the serum had almost entirely lost its power to sensitize corpuscles for the complement-deficient serum, only partial hemolysis resulting when 500 hemolytic units were used. Another portion of the hemolytic

serum was heated at 66°C. for thirty minutes. This serum now gave a titer of 1:2500 with normal guinea-pig serum as complement, while it had entirely lost the power of sensitizing corpuscles for the deficient serum.

This experiment also seems to show that the power of the hemolytic serum to render corpuscles susceptible to lysis by the complement-deficient serum depends upon some factor other than hemolytic units, and that this factor is destroyed by temperatures between 60 and 66°C.

*c. Experiments with an aged hemolytic serum.* Anti-sheep rabbit serum which had a titer of 1:10,000 with serum from normal guinea-pigs as complement rendered corpuscles susceptible to lysis by the complement-deficient serum in doses of 200 or more hemolytic units. After the addition of 0.5 per cent phenol, this serum was stored in an ice-box for eighteen months. The titer of the serum with normal complement was found to be only slightly reduced, but it no longer sensitized corpuscles for the complement-deficient serum, regardless of the number of hemolytic units used. The addition of a small amount of inactivated human serum to the old hemolytic serum restored the property of sensitizing corpuscles for the complement-deficient serum.

*d. Immune hemolytic serum no. 96.* This serum was peculiar in that it failed to cause hemolysis with the complement-deficient serum. Its titer with serum from normal guinea-pigs was 1:20,000. Doses containing 15,000 hemolytic units would not hemolyze 1 unit of corpuscles when the deficient guinea-pig serum was used as complement.

The four observations just described show that the hemolytic serum contained some factor, other than hemolysins which was essential in the reactions with the serum from the complement-deficient guinea-pigs. This factor seems to be the third or thermostable component of complement, since (a) hemolytic sera that are capable of sensitizing corpuscles for the complement-deficient serum lose this power after treatment with yeast, although the hemolytic titer has not been reduced, (b) serum heated above 56°C. loses its power to react with deficient serum while it is still very potent in reactions with normal guinea-pigs

serum, (*c*) on aging, hemolytic sera become non-reactive with the deficient serum but retain the power to react with normal serum and (*d*) a very potent hemolytic serum was encountered which would not sensitize corpuscles for the complement-deficient serum regardless of the number of hemolytic units added to the reaction. It is concluded, therefore, that hypersensitization is not responsible for the phenomenon observed by Ecker and myself, namely, that the complement-deficient serum is lytic for red blood corpuscles, provided that sufficiently large quantites of sensitizing serum are used. The deficient serum becomes active because the hemolytic serum supplies the factor which the deficient serum lacks.

### *Evidence that the third component is quantitatively involved*

The third complement component exists in normal guinea-pig serum in great excess, at least 100 times the quantity necessary to supply its quota to the other components to make the average titer of 100 units per cc. This statement is based on the observation that the deficient complement is made active by the addition of 1 per cent by volume of fresh guinea-pig serum. Since the percentage cannot be carried far below this point, it would seem that the third component is quantitatively involved. The following experiment is designed to give weight to this statement. Three complements were selected as follows: A, deficient guinea-pig serum; B, normal guinea-pig serum; C, complement of the same potency as normal guinea-pig serum, made by adding 5 per cent by volume of B to A.

Complements B and C are of the same potency, and by the addition of 5 per cent of B to A, A becomes as potent as B. If B reactivated A in some way merely by virtue of its quality rather than its quantity, then complement C in the same amount as complement B should reactivate A to the same potency as B and C. As a matter of fact it fails to do so, evidently because it does not contain the requisite amount of the third component.

### *Heredity of the complement-deficient condition*

Moore makes the following statement with reference to the heredity of the complement-deficient condition:

At the time that this deficiency of complement in the serum of some guinea-pigs was discovered, Dr. F. A. Rich, head of the Veterinary Department of the Vermont State Agricultural Experiment Station, and Ramon C. Downing began a breeding experiment with such animals. The results of this experiment have not yet been published; I may say, however, that this deficiency in complement was found to be a heritable condition. As far as I am aware, the complement-deficient guinea-pigs at this Experimental Station are the only ones of the kind in existence; we have been able to multiply them by careful breeding and we have hundreds of them at present.

In a personal communication, Dr. Rich gave us the results of certain crosses which show very clearly that the character behaves as a simple Mendelian recessive. His results were as follows:

709 hybrid offspring all showed normal amount of complement.

1209 $F_2$'s showed on test 302 with low complement and 907 with the usual ranges of titer.

1401 offspring of the hybrid-recessive mating showed on test 711 normal and 690 complement-deficient.

2046 offspring of recessive by recessive mating, all proved deficient for complement.

Dr. Rich adds,

You will see that these are very satisfactory Mendelian ratios but we found in the early part of the breeding that it was absolutely necessary to test the offspring at a very early age (a few days) because of the difficulty of raising the low complement individuals. This lethal nature of the deficient character was evident throughout our six years of breeding these animals.

Our breeding experiments corroborate Dr. Rich's observations as to the behavior of this character in inheritance. A single Mendelian factor is involved. The ratios substantiate this and there is clean-cut segregation of the character involved. Moreover, the extracted deficient are as deficient as the original inbred deficient animals and they breed true to this condition. I have obtained the following results: Twenty-four hybrids from deficient mothers by normal males yielded complement of the same

potency as the serum from normal guinea-pigs. These hybrids when inbred gave 50 in the next generation, 17 of which were deficient and 33 normal. When the hybrids were back crossed to deficient males the ratio was 11 deficient to 15 normal. The offspring from the extracted recessives were all complement-deficient. The sex ratio is near equality and both sexes are equally affected. The inheritance is the same, in so far as the complement is concerned, whether the transmission is through the male or female.

It will be of interest to point out here that the missing factor does not pass through the placenta. A study of the distribution of the character to the offspring from the females of the different combinations makes this point clear. For example, the hybrid offspring from a deficient mother by a normal father were mated. A certain hybrid female gave birth to a litter of 4; 3 were like the normal, and 1 was complement-deficient. The serum of the mother was like that of normal guinea-pigs. Since the deficient offspring was born from a mother whose serum was normal with respect to its complement content and since only a small percentage of normal guinea-pig serum is required to activate the deficient serum, it becomes apparent that the third complement component does not pass from the mother through the placenta to the offspring. The serum of the young was tested during the first forty-eight hours of extrauterine life. It was also found that the missing factor does not pass from the fetus to the mother. Deficient females were mated to normal males. The offspring from this cross are normal and if the third component could pass from the embryo to the mother, the serum of the deficient mother would become active. Our tests showed, however, that there was no change in the complement content of the serum of the deficient mother during the period of gestation or postpartum.

It seems, therefore, that the third complement component arises de novo and whether or not it is present is determined by the inherited genotypical make-up of the individual. We have also observed that the serum of complement-deficient young is not affected by nursing normal mothers.

*General observations on the complement-deficient guinea-pig*

In outward appearance and general behavior the deficient guinea-pigs are not to be distinguished from the normals. They breed readily, the oestrous cycle being about seventeen days and in two cases the deficient mother has given birth to young before she was one hundred days old, indicating that sexual maturity was in this case reached when about thirty days old. This is an infrequent occurrence even among normal guinea-pigs. The young develop rapidly in common with the normal guinea-pigs, often weighing 250 grams or more when one month old. The males reach sexual maturity much later than the females—when they are three or four months of age.

Attention has already been called to the statement by Dr. Rich concerning the lethal nature of the deficient character. One must not conclude from this that the loss of vigor is due to the non-complementing activity as such, although Moore's results would seem to indicate that such were the case. Our ratios were obtained from tests made on young that had survived the weaning time at one month. Despite this fact, the number of deficient is in slight excess of the expectation. There were 10 of the $F_2$ and 5 from the back cross that died before the tests were made. These facts indicate that differential viability played no significant part in the determination of the ratios. My experience with guinea-pigs has shown that they thrive best at a living room temperature. They do not withstand sudden changes in temperature but can be kept at rather cool temperature by acclimatization. This is the experience of many breeders of these animals, although Tresidder (4) is sceptical "about the marked susceptibility of the guinea-pig to cold." My experience with the deficient pigs would indicate that they do quite as well as the normal when kept under ideal conditions, but when subjected to unfavorable temperatures the deficient are the first to succumb.

The hybrids are, apparently, more vigorous than the deficient. Of the 40 pigs received from Dr. Rich in the fall of 1920, only 3 are surviving. Heavy fatality occurred among them during a

cold spell in the fall of 1920 and in the spring of 1921, before adequate housing facilities had been provided. Of the 40 received, all proved deficient except 2. These 2 were found to be heterozygous. One cannot put too much confidence in such isolated observations, but it would seem to be a significant fact that the two heterozygous animals are among the only 3 survivors of the original lot.

The lessened resistance which is associated with the deficiency of complement applies to other unfavorable conditions to which guinea-pigs may be subjected. For example, in collecting blood by cardiac puncture the deficient are more likely to succumb from intrapericardial hemorrhage than are the normals. The fatality for the deficient is fully 20 per cent despite the fact that the technic of the operator has been developed to the point in which a hundred normal animals have been bled in succession with the loss of only 2.

*Incidental observations on the vigor of the stock*

Many mutants are more viable than the parent type, others may be more resistant to some spceific infection. It has been shown that the truncate mutant of *Drosophila melanogaster* (*ampelophila*) does not live as long as the wild type (5c). My experiments (unpublished) have excluded any possibility of this lessened viability being due to the short wings as such. The shortened wing is a certain kind of index to the now unexplored biochemical make-up of the individual; this bears on the question of the complement deficient guinea-pigs. For if the deficient stock can hold its own in a fair way by the side of a stock that has 50 times the complement in its blood, then complement cannot play the rôle in infection and resistance that test tube experiments have led us to believe.

In this connection, it is pointed out that no hesitancy is felt in referring to the deficient guinea-pig as a mutant. This opinion is based on the results of my own work with *Drosophila melanogaster*, *D. confusa*, *D. repleta* and *D. hydei* from which more than 25 mutated stocks have been established, as well as with the behavior of the character at hand. I do not share the opinion

held by Ruediger (6) and so often expressed in the literature as to the extreme variability to be found in the complementing potency of guinea-pig serum. My tests made on several hundred guinea-pigs (excluding pregnant and postpartum females) show that the complement is remarkably constant. Slight variations in technic show great differences in potency of complement even from the same animal. However this may prove to be, it is certain that in the case of the deficient guinea-pig and the normal guinea-pig there is a wide difference which can be measured by immunological methods with as much certainty as the geneticist distinguishes between white and black guinea-pigs in his study of coat colors. The fact that the complement from the hybrid is of the same potency as that from the normal guinea-pig and that only deficient animals and animals of normal titers without intermediate ranges were obtained among the grandchildren from the crosses, would bear out the foregoing conclusions.

Ruediger found that by breeding only guinea-pigs with good complement his collection was greatly improved for complementing purposes within three years. He was of the opinion that the fixability of the complement was influenced by the kind of food the animal received; raw vegetables such as potatoes, cabbage or carrots were suitable. The best results were obtained with mixtures. He further states that "The fixability of guinea-pig complement was much better in winter than in summer, which may be due to the large quantity of grass fed in summer." I have carried out quantitative determinations on the amount of complement in the deficient animals after feeding on a grass diet as well as on diets of raw vegetables. The complement content was not modified as a result of this procedure. In this connection it is pointed out that the non-complementing condition is not incompatible with the production of anti-sheep hemolytic sera. The complement-free animals, moreover, are subject to anaphylactic shock.

## DISCUSSION

Throughout this paper we have referred to the deficient serum as resulting from a missing component. So far as the tests apply

the deficient serum behaves as if this element were entirely missing, which might imply that the material particle or gene upon which the elaboration of this component depends is missing, as the presence and absence theory postulates. In the absence of direct evidence bearing on this case we prefer to believe that the deficient mutation resulted not from a loss but from a change in the germ plasm involving possibly chemical configurations, or physical rearrangements. This conception is based on the evidence found in a study of a system of multiple allelomorphs. This evidence indicates that the mere presence or absence of a gene are not the only two possible states which can exist (5b). In the blood the complementing activity is lost but the material unit upon which this depends may have changed only its physical or chemical state. The fact that guinea-pig complement is inactivated by shaking is not opposed to this last assumption.

Since the serum from the hybrid guinea-pig complemented the same as the normal guinea-pig, it was pointed out that in transmission the complementing condition behaved as a dominant character. It has been shown that 1 per cent by volume of the fresh guinea-pig serum rendered the serum from the deficient guinea-pig like the normal. In other words, the blood serum of a guinea-pig with a small amount of the missing factor functions the same as a serum with a full quota of this factor. This is verified by the in vivo activation experiments. What assurance have we that the hybrid is as well supplied with the third factor as the normal guinea-pigs? Fortunately, the serological titrations enable one to measure the potency of this factor. The hybrids that have been tested in this respect gave exactly the same readings as the normal control before and after heating.

It is well known that when the hemolytic amboceptor is increased, less complement is required to effect a given amount of hemolysis. Thus, according to Noguchi, the amount of complement necessary to hemolyze a unit of red cells is $\frac{1}{3}$, $\frac{1}{5}$, and $\frac{1}{10}$ unit, when 4, 10, and 20 units, respectively of amboceptor are used. I have verified this many times and in addition have found that by increasing the number of amboceptor units to from 200

to 500, the complement may be reduced to one-twentieth of the amount necessary to hemolyze a unit of sheep corpuscles sensitized with 1 unit of anti-sheep rabbit serum, titer 1:10,000. The answer to the question as to why complement and amboceptor are quantitatively interdependent involves a fundamental conception concerning the function of these factors in hemolysis.

Bordet claims that the immune serum renders the corpuscle sensitive to the toxic influence of alexin. With increasing doses of sensitizer the cells are rendered more and more sensitive. Consequently smaller amounts of complement are able to effect their destruction. The phenomenon is claimed to be one of absorption and is dependent upon physical reactions. Our observations on the hemolysis that results with large amounts of amboceptor and the deficient complement show that the immune serum supplies some factor which, coöperating with the small amount of complement, makes hemolysis possible and that the quantitative interdependence that exists between amboceptor and complement is not to be explained entirely on the assumption that the corpuscles are rendered more vulnerable to the complement in consequence of supersensitization. The fact that a unit of cells charged with 2 units of amboceptor becomes susceptible to lysis by 0.02 cc. of deficient guinea-pig serum on the addition of 0.0002 cc. of heated human serum, while in the absence of the human serum 0.8 cc. of deficient serum is required, must be attributed to some property of the human serum other than its sensitizer content. The result would seem to find its best interpretation on the assumption that the human serum supplies a connecting bond. The bond in this case is the third complement component.

Our knowledge of heritable characters affecting the blood of animals is limited to man. The four well defined groups into which individuals of this species can be classified with respect to iso-agglutination finds a ready explanation in terms of the segregation and recombination of two Mendelian factors. The other condition known as hemophilia is a sex-linked recessive character involving some agent concerned with the coagulation of the blood. The new mutation in the guinea-pig furnishes an excel-

lent example of a simple recessive Mendelian unit. In this case the gene is identified with the third complement component, one of the agents concerned in hemolytic reactions. That the deficient gene finds identical expression in the soma of all the deficient animals (or at least affects one part of the complementing mechanism in common) is shown by the fact that in many experiments in which the fresh serum from the different deficient animals was mixed, the complementing potency was not restored.

It is noted that the germ cells of the hybrid bearing "deficiency" maintain their individuality with respect to this character despite the fact that throughout the period of their reproduction and growth they have been bathed and nourished by a liquid rich in the very element which nature designed should be held and produced normally by the developed zygote. This fact serves to emphasize the complete protection of the germ cells, not only from the outside world but even from the fluids in which they are bathed. The evidence brings to light a relation between the blood stream and the germ cells which bears on a recent claim as to the inheritance of acquired characters made by Guyer and Smith (7), (8). These investigators found an eye defect in some young rabbits born from a mother that had been treated during the gestation period with an immune anti-rabbit-lens chicken serum. This eye defect was transmitted by the affected individual to succeeding generations. These writers contend that the defect was induced by the "sensitized" serum, and that the germ plasm had been specifically changed. Their argument rested on the fact that the germ cells are dependent on the blood stream for their nutrition and development; and since the blood stream can be readily charged with antibodies of specific affinities, the ideal situation was presented for the change to take place.

Guyer's results, taken at their face value, would seem to indicate that the germ cells of the developing young were changed at the same time as the lens in the eye. If this is true the germ cells of the mother should have been affected in like manner.

Since the character apparently behaves as a recessive the question could have been tested by breeding the treated mother to a defective male. Guyer does not indicate the results from any such combinations.

With the complement-free guinea-pig we have demonstrated the absolute lack of influence between the third complement component and the gene for this character in the germ plasm. In so far as this case serves as an index of the relation existing between the blood stream and the germ plasm we feel that any claim as to the inheritance of an acquired character as a result of changes induced through the blood stream should not be accepted without rigorous proof. The facts here presented would seem to weigh heavily against the interpretation which Guyer has placed on his results.

A corollary to the foregoing propositions that has often been raised in connection with Mendelian work has related to the possibility of a gene being contaminated as a result of its association in the hybrid with another gene. If it were possible for contamination to occur as a result of the coexistence of two genes as many selectionists hold, the hybrid guinea-pig would seem to offer the ideal place for such an event to occur. And yet we find genes of this hybrid segregated into the same classes that entered into its make-up without these units having influenced each other in any way.

It would seem that genes are fairly stable entities; they apparently have inherent fixed individualities. There is no more reason to believe that the gene for complement has been modified as a result of its coexistence with its deficient allelomorph than that the chemical properties of chlorine have entirely changed as a result of its having coexisted with sodium in common salt.

## SUMMARY

1. A stock of guinea-pigs obtained from the Vermont Agricultural Experiment Station through the courtesy of Dr. F. A. Rich has shown a very marked deficiency in complement. Our results in this respect are in complete agreement with those obtained by Moore and Downing.

2. Our evidence substantiates the claim of Coca that in these pigs the third (heat resistant or yeast absorbed) complement component is missing.

3. The deficient serum may be activated for hemolytic tests by the addition of very small amounts of fresh human, guinea-pig, dog, cat, or rabbit serum, but not with the fresh serum of the ox, pig, duck, chicken, pigeon, sheep, frog, goat or mouse. Heated human serum is a potent activator. Certain sera have supplied as many as 5000 activating units per cubic centimeter.

4. The deficient serum is activated in vivo by injections of the fresh or heated serum from the normal guinea-pig or man. The activation is instantaneous after intravenous injections, with subcutaneous or intraperitoneal injection the activation is from four to eighteen hours later. The animal returns to its previous non-complementing state after three or four days.

5. Complement-deficient serum is lytic for red corpuscles provided that large quantities of sensitizing serum are used. The deficient serum becomes active under these conditions because the sensitizing serum supplied the factor which the deficient serum lacks. This factor seems to be the third or thermostable complement component, since (*a*) hemolytic sera that are capable of sensitizing corpuscles for the complement-deficient serum lose this power after treatment with yeast, although the hemolytic titer has not been reduced, (*b*) serum heated above 56°C. loses its power to react with deficient serum while it is still very potent in reactions with normal guinea-pig serum, (*c*) on aging, hemolytic sera become non-reactive with the deficient serum but retain the power to react with normal serum, and (*d*) a very potent hemolytic serum was encountered which would not sensitize corpuscles for the complement-deficient serum regardless of the number of hemolytic units added to the reaction. This evidence denies Ecker's claim to the effect that the susceptibility of the corpuscles to the lytic action of the deficient serum is due to supersensitization per se.

6. The third complement component would seem to share certain properties usually attributed to enzymes since it brings about changes out of all proportions to the amount involved. This component although produced by a living organism, is not in itself living in the sense that it possesses independent power of growth and reproduction. It effects changes not merely by virtue of its quality but also by virtue of its quantity.

7. The complement-deficient guinea-pig would seem to hold its own quite as well as the normal when kept under ideal conditions but succumbs more readily when the environment is adverse.

8. The complementing condition of the deficient guinea-pig is not modified as a result of keeping the animal on a diet of grass, raw vegetables, etc.

9. The complement-deficient condition behaves in heredity as a simple recessive Mendelian unit which gives clean-cut segregation from its normal allelomorph.

10. The third complement component does not pass through placenta from mother to offspring. Neither does it pass from the fetus to the mother. The third component is of autochthonic origin and whether or not it is present is determined by the inherited genotypical make-up of the zygote.

11. The recent claim of Guyer to the effect that acquired characters impress themselves on the germ plasm by way of the blood stream finds no support in light of the clear-cut lack of influence which is shown to exist between the third complement component in the blood and the gene for this character in the germ plasm of the guinea-pig.

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# STUDIES IN SPECIFIC HYPERSENSITIVENESS

## RELATIVE SUSCEPTIBILITY OF THE AMERICAN INDIAN RACE AND THE WHITE RACE TO POISON IVY

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In a previous paper (1) it was pointed out that certain publications seemed to indicate that some of the forms of hypersensitiveness in human beings (asthma, dermatitis venenata) are rare among the American Indians. It was thought that investigation of this apparent radical difference might produce some information regarding the nature of the different forms of specific human hypersensitiveness as well as some indication of the relation of the American Indian to the other human races. An experimental study of the occurrence of serum disease in 26 volunteer full-blooded American Indians indicated that the Indian race is much less susceptible to that condition than is the white race.

That the American Indian is only slightly susceptible to poison ivy was indicated by the report of Lain (2) in which he states that though the Indians frequently apply quite freely on their faces and other exposed parts paints and dyes made from wild plants, berries, etc., the skin of the Indian is apparently almost immune to the poison ivy. Answers to a questionnaire, as previously cited (1), from eleven physicians and one superintendant of an Indian School tended to confirm this view.

A means of eliminating the variable factor of natural contact with the exciting agent and at the same time of making a direct determination of the relative susceptibility of the two races was afforded by the convenient "patch test" of Dr. Cooke (unpublished clincial studies) which has been shown by Coca (3) and by

Spain (4) to be a reliable test for individual susceptibility to ivy poisoning. Hence it seemed desirable to use this method for the further comparative study of the two races.

The test was carried out by applying the resinous extract of poison ivy leaves (*Toxicodendron radicans* L.) to a small area of the skin on the anterior surface of the forearm. The area was protected with adhesive. Since the extract is quite soluble in the adhesive material, a reversed square of adhesive was interposed so that only the canvas came in contact with the extract. The adhesive patch was kept in place at least two days.

The extract was prepared by immersing young ivy leaves in chloroform or toluol, in both of which the active agent is soluble. After twenty-four hours the leaves were removed by filtration through paper and the filtrate was evaporated in a shallow dish before an electric fan until a semi-solid, sticky residue remained. Less than two months elapsed between the preparation of the extract and its use. The same preparation was used throughout.

The test was carried out on 227 American Indians from 32 tribes, Navajo, Pueblo, and Apache predominating. Only individuals who were listed in the government records as full-blooded were selected, and some who were so listed were rejected because it was suspected that they might be breeds. The list, then, comprises only full-blooded Indians, so far as it is possible to determine, and probably represents a high percentage of purity. The ages ranged between fourteen and about twenty-four years. The members of the white race who were tested were mainly students in the classes of Dr. N. P. Sherwood at the University of Kansas. No white individual under fourteen years of age was tested.

In table 1 are listed the results of tests with poison ivy extract on Indians and whites. Definite erythema with itching was considered a positive reaction. Observations were made as nearly as possible on the fifth day after applying the extract.

Table 1 shows that of 227 American Indians tested with poison ivy extract 56 per cent gave positive reactions; and of 46 white individuals 58.8 per cent gave positive reactions.

These tests, then, indicate that the American Indian race differs slightly, if at all, from the white race in susceptibility to poison ivy.

It is a pleasure to express our indebtedness to Mr. Clyde M. Blair, superintendent of the Chilocco Indian School, Chilocco, Oklahoma; James Jonas, Ray Colglazier and Miss McCormick, disciplinarians; Rose Daugherty, superintendent of guest quarters at Chilocco Indian School; to Mr. Reuben Perry, superintendent of United States Indian School, Albuquerque, N. M.; Mr. Vigil and Mrs. Hickson, disciplinarians: to Mr. H. B. Pearis, superintendent at Haskell Institute, Lawrence, Kansas; Dr. Lawrence

TABLE 1

| | DAY OBSERVED | NUMBER TESTED | POSITIVE REACTIONS | PERCENTAGE POSITIVE | OBSERVED BY |
|---|---|---|---|---|---|
| | | | | *per cent* | |
| Chilocco Indian School.......... | 4 | 50 | 36 | 72 | E. F. M |
| School at Albuquerque.......... | 5 | 80 | 32 | 40 | A. M. W. |
| Haskell Institute................ | 4 or 5 | 97 | 59 | 60.8 | O. D. |
| All Indians.................... | | 227 | 127 | 56 | |
| Whites.......................... | 5 or 6 | 46 | 27 | 58.8 | O. D. |

White and Dr. A. J. Anderson, physicians; Mr. Peru W. Farver and Miss Anne F. Ritter, disciplinarians at Haskell: and also to the volunteer Indian students of the various schools, for the splendid coöperation which they gave in the execution of this work as well as in the blood grouping tests reported in another paper (5).

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# ON THE BIOCHEMICAL RACE-INDEX OF KOREANS MANCHUS AND JAPANESE

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The studies of Landsteiner (1), Decastello and Sturli (2), Jansky (3), Moss (4), von Dungern and Hirschfeld (5) and others, have established the fact that there are four different groups of human individuals with respect to the isoagglutinating and isoagglutinable blood elements. In 1919, L. Hirschfeld and H. Hirschfeld (6) studied a number of soldiers belonging to the Allied Armies in Macedonia, and found that there was a remarkable difference in the blood grouping in the different races. The proportion of A (in the corpuscles of groups II and IV[1]) to B (in the corpuscles of groups III and IV); i.e., $\frac{A+AB}{B+AB}$ or $\frac{II + IV}{III + IV}$ was called by them "the biochemical race-index," and the race, in which the index amounted to more than 2.0 was called "of European type," and that, from 2.0 to 1.0, "of intermediate type" and that, less than 1.0 "of Asio-African type."

In 1921, F. Verzar and O. Weszaczky (7) discovered in Hungary that the biochemical race-index of persons descended from the same racial origin was practically the same and that influences such as geography, climate or food could never alter the index.

The following study was made to find the biochemical race-index in Koreans, Manchus and Japanese. The method of inves-

[1] In this article the isoagglutinins and isoagglutinable elements have been designated with the letters originally given them by Landsteiner and recognized in the later extensive studies of von Dungern, of the Hirschfelds and of Ottenberg.

The group numbering is that of Jansky, which has been officially adopted by the American Association of Pathologists, the Society of American Bacteriologists, and the American Association of Immunologists.

tigation was as follows: The a and b sera were obtained from a group III and a group II individual respectively. To 9 cc. of each serum 1 cc. of 3 per cent saline solution of carbolic acid was added in order to prevent the production of bacterial hemagglutinin. These were used as standard sera. Then, a few drops of blood taken from the ear lobe of the persons for test were added to about 2 cc. of saline solution containing 2 per cent of sodium citrate, and the blood was washed twice with saline solution. A 20 per cent suspension of the blood corpuscles was made. Two drops of the a and b sera were separately mixed with one drop of the corpuscle suspension. In case agglutination occurred in both mixtures, the blood of the person was grouped as AB. In case no agglutination occurred, the blood was grouped as O. And in case agglutination occurred in the a serum but not in the b, the blood was grouped as A and, if reverse, as B. The age of the persons that were studied was above ten. The standard sera were often examined with my own blood to see whether they were intact or not. The result of the examination of Koreans is shown in table 1.

Judging from the index of table 1, Koreans are of intermediate type (of Hirschfeld), though not far from Asio-African type.

The result of the study of the Manchus (including people of Fengtien, Kirin and Hei-Lun-Kiang) is shown in table 2.

From table 2, it is evident that the Manchus belong to Asio-African type.

The result of the study of the Japanese is shown in table 3.

From table 3, it is evident that the Japanese belong to an intermediate type that is quite different from that of the Koreans and Manchus. It is noteworthy, however, that the biochemical race-index gradually decreases in Japan from south to north. The meaning of this fact will become clear, if the race-index is examined in the southern part of Korea, the northern part of Manchuria, the districts of Mongolia and Siberia, since the Japanese have some racial relation to the people in those places.

The blood of some people of Chih-Li, Shan-Tung, Hu-Nan Ho-Nan, Kiang-Su, and An-Hweih was also examined by the author in Moukden the result of which is here preliminarily reported (table 4).

TABLE 1

*Koreans*

| NAME OF PLACE | | TOTAL | A(II) | | B(III) | | AB(IV) | | O(I) | | $\frac{A+AB}{B+AB}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Number | Per cent | Number | Per cent | Number | Per cent | Number | Per cent | |
| Seoul | Total.... | 179 | 64 | 35.75 | 45 | 25.13 | 26 | 14.52 | 44 | 24.58 | 1.26 |
| | Man..... | 121 | 43 | 35.53 | 27 | 22.31 | 21 | 17.35 | 30 | 25.61 | 1.33 |
| | Woman | 58 | 21 | 36.20 | 18 | 31.20 | 5 | 8.62 | 14 | 24.13 | 1.13 |
| Phyeng-yang | Total.... | 184 | 55 | 29.89 | 51 | 27.71 | 20 | 10.86 | 58 | 31.52 | 1.05 |
| | Man..... | 100 | 30 | 30.00 | 33 | 33.00 | 11 | 11.00 | 26 | 26.00 | 0.93 |
| | Woman | 84 | 25 | 29.76 | 18 | 21.42 | 9 | 10.71 | 32 | 38.09 | 1.25 |
| Sum total............ | | 363 | 119 | 32.78 | 96 | 26.44 | 46 | 12.67 | 102 | 28.09 | 1.16 |

TABLE 2

*Manchus*

| NAME OF PLACE | | TOTAL | A(II) | | B(III) | | AB(IV) | | O(I) | | $\frac{A+AB}{B+AB}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Number | Per cent | Number | Per cent | Number | Per cent | Number | Per cent | |
| Moukden | Total........ | 199 | 53 | 26.63 | 76 | 38.19 | 17 | 8.54 | 53 | 26.63 | 0.75 |
| | Man......... | 127 | 33 | 25.98 | 48 | 37.79 | 12 | 9.44 | 34 | 26.77 | 0.75 |
| | Woman...... | 72 | 20 | 27.77 | 28 | 38.88 | 5 | 6.94 | 19 | 26.38 | 0.75 |

TABLE 3

*Japanese*

| NAME OF PLACE | TOTAL | A(II) | B(III) | AB(IV) | O(I) | $\frac{A+AB}{B+AB}$ |
|---|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* | |
| Nagano.............. | 353 | 40.5 | 16.0 | 20.0 | 24.0 | 1.68 |
| Sendai............... | | 37.0 | 19.2 | 11.3 | 32.5 | 1.58 |
| Fukuoka............. | 170 | 45.3 | 20.2 | 10.6 | 24.1 | 1.82 |
| Sum.............. | | 40.9 | 18.4 | 13.9 | 26.8 | 1.69 |

The province of Nagano (studied by Hara and Kobayashi (8)) is situated in the middle part of Japan.

Sendai (studied by Matubara (9)) is located in the northern part of Japan.

Fukuoka (studied by the author) is situated in the southern part of Japan.

Among the Central Chinese, 33 persons of Shan-Tung and 12 persons of Hu-Nan, Ho-Nan, An-Hwei and Kiang-Su were examined, with the result shown in table 5.

Although I can not draw a final conclusion from these results, as the number of persons examined was very small, the race-index seems to increase from the Manchus (north) to the Central Chinese (south). A future study on the race-index of more persons of Central Chinese origin may confirm this fact and then the race-index of Chinese and Japanese can be definitely compared.

TABLE 4

*Chinese*

| NAME OF PLACE | TOTAL | A(II) | | B(III) | | AB(IV) | | O(I) | | $\frac{A+AB}{B+AB}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Number | Per cent | Number | Per cent | Number | Per cent | Number | Per cent | |
| Chih-Li................ | 35 | 10 | 28.57 | 9 | 25.71 | 3 | 8.57 | 13 | 37.14 | 1.18 |

TABLE 5

*Chinese*

| NAME OF PLACE | TOTAL | A(II) | | B(III) | | AB(IV) | | O(I) | | $\frac{A+AB}{B+AB}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Number | Per cent | Number | Per cent | Number | Per cent | Number | Per cent | |
| China.................. | 45 | 17 | 37.77 | 11 | 24.44 | 3 | 6.66 | 14 | 31.11 | 1.42 |

To summarize the foregoing studies, the blood type of the Koreans is quite near the Asio-African, as it shows a very low intermediate type while that of Manchus is Asio-African. It is evident, therefore, that there is a marked difference between the race-index of the Japanese and that of the Koreans or Manchus.

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# THE VACCINE TREATMENT OF ASTHMA[1]

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The literature dealing with human sensitization and allergy contains an abundance of articles dealing with the differential diagnosis of the different varieties of asthma and with broad suggestions as to their treatment, but the reports showing the actual results of treatment, particularly of vaccine treatment, are few.

In 1919, Walker (1) stated the results in a total of 178 patients treated with vaccines; of these 28 were sensitive to bacterial proteins and after treatment with a stock vaccine made from the corresponding organism, 75 per cent were relieved of asthma and 21 per cent were improved. Of the remaining 150 patients non-sensitive to bacterial proteins 40 per cent were relieved and 20 per cent were improved. Most of these were treated by an autogenous vaccine. This is the largest reported series in the literature. Montgomery and Sicard (2) treated 16 cases with autogenous vaccines and 75 per cent were cured. Hutcheson and Budd (3) using a vaccine made by incubating a mixture of 1 cc. of washed sputum with 10 cc. of serum broth which was then killed and standardized, treated 20 cases, of which 12 were completely relieved and 5 were greatly improved. Rogers (4) writing in the British Medical Journal claims good results in over 52 per cent of his 40 patients. He also used an autogenous vaccine made from the sputum. Wickett, Corley and Connell (5) treated 13 cases with autogenous vaccines, 10 of which showed complete relief.

The chief reason why those treating asthmatics have hesitated to publish definite results of their treatment is the great difficulty

[1] From the Medical Services of the Massachusetts General Hospital.

of determining whether or not any one particular measure is responsible for the change in the patient's condition. Bronchial asthma is a disease characterized by attacks which recur and which clear up slowly or suddenly. These attacks may be of long duration or of short duration. Their cause may be known or not. When a patient first reports for treatment it is difficult or almost impossible for the doctor not to give him advice or drugs which alone may have a definite effect upon the disease. A week in bed in the hospital ward often results in a virtual cure. The rest and easy breathing during the stay improves the patient's general condition to such an extent that unless he is sensitive to some dust, he can get along at home often for considerable periods without much asthma. How frequently potassium iodide is prescribed on the day of the first visit and of the first dose of vaccine and how frequently on the same day the patient's diet is adjusted and he is advised to rest at least one hour at noon time!

Referring more definitely to vaccines, other factors may be of importance. Several patients are quite delighted with the results obtained during this winter of 1922–1923. According to their history they have in the past been free of asthma during the winter. They were treated this year only because during the winter of 1921–1922, asthma hung on until Thanksgiving, a complication which has this year been prevented. Differences in the particular winter season may be a factor. A trip to Florida often makes the patient feel that vaccines are "wonderful." From the above it is evident that there is no good check upon the results of vaccine treatment. It is with these facts in mind that the following report is presented.

Asthmatics in this series have been selected for vaccine treatment mainly by exclusion. Those treated in this way are those in whom foreign proteins are not a factor, who have no focus of infection, whose diet and whose habits and mode of life are already so regulated as to preserve the general health as far as possible. They may be divided into three clinical groups: first, those with chronic pulmonary emphysema including a barrel-shaped chest, hyperresonance and more or less cyanosis;

second, those in whom a chronic cough is the predominant factor, the wheezing being apparently a secondary matter, and third (the largest group), those with asthma but without permanent emphysema.

The following discussion of results does not distinguish between the clinical groups although the tables will show the age of each patient and the group to which he belongs. So far there is no way of telling in advance whether vaccines are going to help a given patient or not.

Treatment may be with stock vaccines or with autogenous vaccines and the choice is undoubtedly important but as we know so little of the mechanism involved, the choice of method is so far not defined.

Autogenous vaccines are made from the sputum as follows: Fresh sputum is collected in a sterile bottle. Suitable thick portions of this sputum are teased out and are then broken up and mixed on the edge of a rabbit-blood agar plate. From this edge streaks with a clean sterile loop are made across the plate following the "sun burst" pattern. In this way the colonies are separated so that they can be easily "fished" and transplanted to the sections of a second plate. The growth on this second plate can be observed for its purity and from it an abundance of material is available for further transplants. The vaccine for treatment is made by inoculating a tube of dextrose broth from the second plate and washing the twenty-four-hour growth in this broth three times with salt solution containing 0.5 per cent carbolic acid. The growth is then suspended in this carbolised saline and is killed by heating to 56°C. for one hour. The vaccines have been made to contain approximately two billion organisms per cubic centimeter although no attempt at accurate standardization has been attempted. It is easy to make from 4 to 6 different vaccines for each patient and the question of selecting the proper vaccine for treatment then presents itself.

In 1920 one of us (6) stated that vaccines in asthma were successful in accordance with the production of a local reaction following their injection. At that time the use of intradermal

tests as a method of selecting the proper vaccines was advocated. Since then this method has been found unsatisfactory for the reason that certain vaccines show large local reactions in each and every individual and consequently the present technic is to inject the patient subcutaneously with small equal doses of each and every vaccine made and to study any local reactions which appear in twenty-four hours; the degree of redness and of swelling indicates whether or not the corresponding vaccine should be used for further treatment. The test doses have been set arbitrarily at 0.25 cc. corresponding to about five hundred million organisms.

The treatment itself has consisted of further doses of vaccine given at intervals of five to seven days. The size of these doses has been determined entirely according to the amount of local reaction in the arm which followed the previous dose. The object has been to inject such a quantity of vaccine that a local reaction about the size of a silver dollar is produced each time. When the previous reaction was less than a dime the next dose was usually doubled but where the previous local reaction was larger than a silver dollar, the next dose has been increased less or not at all.

While it has not been uncommon to reach a dose of two billion organisms on the third injection, such a dose has in most cases not been reached until the fifth or sixth injection. The series of treatments has been continued until a very definite improvement in the patient's condition became evident or until the total dosage reached 1.5 cc. or two billion organisms without any such change in the patient's condition. In the event that 6 doses of good size have been given without benefit and without local reactions, experience has shown that further treatment with this particular organism is of no avail.

On the other hand where initial improvement has been manifest, recent experience has justified the continuation of vaccine treatment over considerable periods of time. It is felt that relapses so common in the past can be prevented. So after a preliminary intensive series of 6 to 10 inoculations, the intervals between the doses have been gradually lengthened up to a

month, provided only that the patient has remained free of asthma during the interval. It has been found that patients who are doing well will report at the end of a month saying that they are not entirely free of a slight wheeze or cough. We feel that 1 or 2 additional doses given at this time and at other long intervals serve to maintain the improvement already gained.

TABLE 1

*Treatment of asthma with vaccines; total results in relation to local reactions following treatment*

| RESULTS OF TREATMENT | AUTOGENOUS | | | | STOCK | | GRAND TOTALS |
|---|---|---|---|---|---|---|---|
| | Organism predominant | | Organism not predominant | | | | |
| | Local re-action | No local re-action | Local re-action | No local re-action | Local re-action | No local re-action | |
| ++++ ("cured") | 2 | 0 | 1 | 1 | 5 | 0 | |
| +++ (much improved) | 9 | 0 | 8 | 3 | 11 | 0 | |
| ++ (improved) | 5 | 0 | 2 | 0 | 6 | 0 | |
| + (temporary relief) | 6 | 0 | 12 | 0 | 6 | 0 | |
| Total improved | 22 | 0 | 23 | 4 | 28 | 0 | = 77 |
| Not improved | 0 | 7 | 5 | 29 | 1 | 12 | = 54 |
| Total cases | 22 | 7 | 28 | 33 | 29 | 12 | =131 |
| | 29 | | 61 | | 41 | | |

Figures show number of cases treated.

There is no question but that successful results following vaccine treatment occur only in those cases in whom the test dose and the subsequent inoculations are accompanied by a considerable local reaction in the patient's arm, which consists usually of a red swollen area at least 1½ inches in diameter.

The charts and tables bring out the relation between good results and local reactions following the therapeutic doses.

Table 1 gives the results in the entire series. To the left are the results following autogenous vaccines. When there is a predominating organism, a vaccine from which produces a local reaction there is a good result in each case, but if the pre-

dominating organism fails to give a local reaction, good results are absent. Even when there is no predominating organism a local reaction following treatment is accompanied by good results in most of the cases whereas without any local reaction the results are usually bad.

The same principle holds with regard to stock vaccines. On the right of table 1 we see that where a local reaction followed the treatment, the results are good in all but 1 of the 29 cases, but where there was no local reaction, none of the patients were benefited.

Table 2 shows the results in the 22 cases in which a local reaction followed treatment with a vaccine made from the predominating organism. Note that this table includes 4 patients with emphysema; that it includes 6 patients who were over fifty years old at the time of treatment and that it includes 4 children. The variety of organisms which predominated is striking. In several patients, other autogenous vaccines were used at the same time as the one made from the predominating organism, usually because the local reactions produced by this other vaccine were also large. The number of treatments varied. A question mark indicates that they were given by other doctors. While all these patients were improved, the degree of their improvement was by no means always marked. Two are apparently "cured." Both were boys of eleven who were treated with a vaccine made from predominating non-hemolytic streptococci. One (G. U.) received through the winter of 1919–1920 a total of 10 doses and 4 additional doses were given during the spring. During the past three years he has had no asthma at all, whereas before the treatment, colds would occur each month and would be accompanied by asthma severe enough to keep him in bed for periods of from three to seven days. The other boy (D. E.) was treated during the early part of 1919 and again in 1920. Each time the doses stopped his attacks. In 1922 he had two bad colds and was given 4 doses of a vaccine made from another non-hemolytic streptococcus which was again predominant in his sputum. With this relapse, however, the asthma was not severe. This year (1923) he has been well.

TABLE 2

*Predominant organism; local reactions; improved*

| | INITIALS | AGE | DIAGNOSIS | PREDOMINANT ORGANISM | OTHER VACCINES USED AT SAME TIME | NUMBER TREATMENTS | RESULT* |
|---|---|---|---|---|---|---|---|
| 1 | G.W.B. | 53 | Cough | Pneumococcus type III | Yes | (?) | ++ |
| 2 | P.S. | 38 | Emphysema | Green streptococcus† | Yes | 4 | + |
| 3 | G.S. | 55 | Bacterial asthma | Non-hemolytic streptococcus | Yes | 3 | +++ |
| 4 | M.D. | 47 | Emphysema | Hemolytic streptococcus | Yes | 6 | ++ |
| 5 | F.C.D. | 35 | Bacterial asthma | Pneumococcus type (?) | Yes | (?) | + |
| 6 | R.D. | 9 | Bacterial asthma | Hemolytic streptococcus | No | (?) | +++ |
| 7 | J.W.C. | 43 | Bacterial asthma | Green streptococcus | No | 7 | +++ |
| 8 | L.A. | 45 | Bacterial asthma | Gram-negative bacillus‡ | No | 5 | +++ |
| 9 | M.S.K. | 8 | Bacterial asthma | *Staphylococcus albus* | No | (?) | ++ |
| 10 | M.L. | 25 | Bacterial asthma | Hemolytic streptococcus | No | 8 | +++ |
| 11 | G.U. | 11 | Bacterial asthma | Non-hemolytic streptococcus | No | 14 | ++++ |
| 12 | N.R.C. | 51 | Bacterial asthma | Non-hemolytic streptococcus | No | 6 | + |
| 13 | C.F.F. | 49 | Bacterial asthma | *Staphylococcus aureus* | Yes | 15 | + |
| 14 | L.B. | 57 | Bacterial asthma | Hemolytic streptococcus | Yes | 5 | + |
| 15 | R.E.C. | 36 | Cough | Hemolytic streptococcus | No | (?) | + |
| 16 | E.A. | 50 | Bacterial asthma | Hemolytic streptococcus | No | 17 | +++ |
| 17 | M.P. | 44 | Emphysema | Hemolytic streptococcus | No | (?) | ++ |
| 18 | D.E. | 11 | Bacterial asthma | Non-hemolytic streptococcus | No | (?) | ++++ |
| 19 | J.F. | 19 | Bacterial asthma | Non hemolytic streptococcus | Yes | 5 | ++ |
| 20 | R.C. | 38 | Emphysema | Non-hemolytic streptococcus | Yes | (?) | +++ |
| 21 | J.W.G. | 41 | Bacterial asthma | Green streptococcus | No | 25 | +++ |
| 22 | N.B. | 52 | Bacterial asthma | Green streptococcus | No | (?) | +++ |

* Results: ++++, "cured"; +++, much improved; ++, improved, and +, temporary relief.

† Green streptotoccus indicates a streptococcus producing a green zone on a blood agar plate.

‡ This Gram-negative bacillus grows readily in all media. It is not the influenza bacillus. It has been mentioned by Walker (1) and other investigators in this field.

TABLE 3

*Predominant organism; no local reaction; not improved*

| | INITIALS | AGE | DIAGNOSIS | PREDOMINANT ORGANISM | OTHER VACCINES USED AT SAME TIME | NUMBER TREATMENTS | RESULT |
|---|---|---|---|---|---|---|---|
| 1 | C.P. | 29 | Bacterial asthma | Non-hemolytic streptococcus | No | 5 | Not improved |
| 2 | A.F. | 14 | Cough | Influenza bacillus | Yes | 5 | Not improved |
| 3 | L.B. | 36 | Bacterial asthma | Non-hemolytic streptococcus | Yes | 8 | Not improved |
| 4 | W.L.S. | 42 | Bacterial asthma | Green streptococcus* | Yes | 6 | Not improved |
| 5 | P.D. | 23 | Bacterial asthma | Green streptococcus | Yes | 4 | Not improved |
| 6 | J.S.F. | 42 | Bacterial asthma | Green streptococcus | Yes | 3 | Not improved |
| 7 | G.N. | 4 | Bacterial asthma | Green streptococcus | No | 3 | Not improved |

* See under table 2.

TABLE 4

*No predominant organism; local reaction; improved*

| | INITIALS | AGE | DIAGNOSIS | ORGANISMS USED | NUMBER TREATMENTS | RESULT* |
|---|---|---|---|---|---|---|
| 1 | M.B. | 30 | Bacterial asthma | Non-hemolytic streptococcus | 3 | + |
| 2 | J.C. | 41 | Bacterial asthma | Non-hemolytic and green streptococcus | 15 | +++ |
| 3 | P.F.C. | 45 | Bacterial asthma | Hemolytic and green streptococcus | 7 | +++ |
| 4 | G.G. | 10 | Bacterial asthma | Green streptococcus | (?) | +++ |
| 5 | A.G. | 34 | Bacterial asthma | Staphylococcus and green streptococcus | 5 | + |
| 6 | H.W.H. | 60 | Emphysema | Non hemolytic and green streptococci | (?) | + |
| 7 | M.L.K. | 40 | Emphysema | Non-hemolytic streptococcus | (?) | ++ |
| 8 | M.K. | 6 | Bacterial asthma | Pneumococcus | (?) | +++ |
| 9 | P.J.M. | 45 | Emphysema | Non-hemolytic and green streptococci | 10 | + |
| 10 | P.M. | 33 | Emphysema | Green and hemolytic streptococci | (?) | + |
| 11 | E.H.O. | 21 | Bacterial asthma | Mixed | (?) | + |
| 12 | J.M.P. | 43 | Emphysema | Mixed | (?) | ++ |
| 13 | E.R. | 44 | Emphysema | Hemolytic and non-hemolytic streptococcus | (?) | + |
| 14 | E.S. | 30 | Emphysema | Hemolytic, non-hemolytic and green streptococci | (?) | + |
| 15 | M.S. | 13 | Bacterial asthma | Hemolytic streptococcus | 8 | +++ |
| 16 | G.S. | 52 | Emphysema | Hemolytic streptococcus | 14 | +++ |
| 17 | G.E.S. | 13 | Bacterial asthma | Mixed | 4 | ++++ |
| 18 | J.F.S. | 22 | Bacterial asthma | Non-hemolytic and hemolytic streptococcus | 8 | +++ |
| 19 | T.H.S. | 45 | Bacterial asthma | Green and non-hemolytic streptococci | 4 | + |
| 20 | G.T | 28 | Bacterial asthma | Hemolytic and non-hemolytic streptococcus | (?) | + |
| 21 | M.L.W. | 26 | Bacterial asthma | Hemolytic streptococcus | 6 | + |
| 22 | E.W. | 34 | Cough | Hemolytic and green streptococci | 7 | +++ |
| 23 | C.M.W. | 50 | Emphysema | Staphylococcus | (?) | + |

* See under table 2

Nine patients were much improved. In 5 others, however, the improvement was only slight and in 6 the improvement was temporary—real success therefore in only half the number.

Table 3 shows the type of cases and the bacteriology in 7 patients who were treated with a vaccine of their predominating organism without a local reaction and without improvement.

Table 4 shows 23 patients who were treated with one or more autogenous vaccines made from organisms which were not predominant in the sputum but these vaccines did produce a local reaction and ultimately brought about considerable relief. The patient who was "cured" was one of the early cases (G. E. S.) a colored boy of thirteen who in October, 1919, received only 4 treatments with a mixed vaccine made by planting his sputum on a Loeffler's blood serum slant and using the entire growth. He has had no asthma whatever since the treatment. Eight other patients were much improved, but in 14 the improvement was only slight or temporary so that only 39 per cent were really benefited. As stated above, however, it is not impossible that had the treatment been carried on over a longer period, some of the results which have appeared to be only temporary, might have been made permanent.

These 23 cases treated with an autogenous vaccine without a predominating organism include 9 with emphysema, 3 of whom were over fifty years old and 1 of whom did well. They include 4 children, 1 of whom has been "cured" as noted above and the others markedly benefited. It is interesting that the types of organisms used for treatment in all except 3 cases were streptococci.

The fact that 4 patients have been very definitely relieved by vaccines which produced very little if any local reaction after injections is, in our experience remarkable (table 5). The patient who was "cured" was a slender delicate youth of twenty who was treated in October, 1922, with a green-producing streptococcus which was not predominant—the treatment being carried on because of his definite and continued improvement. During the treatment he stopped having asthma and gained over 10 pounds in weight. The word "cured" is, however,

TABLE 5

*No predominant organism; no local reaction but improved*

| | INITIALS | AGE | DIAGNOSIS | ORGANISM USED | NUMBER TREATMENTS | RESULT* |
|---|---|---|---|---|---|---|
| 1 | T.J.B. | 60 | Emphysema | Green and non hemolytic streptococcus | 7 | +++ |
| 2 | S.P. | 20 | Bacterial asthma | Green streptococcus | 8 | ++++ |
| 3 | A.P. | 38 | Bacterial asthma | *Staphylococcus aureus* and green streptococcus | 14 | +++ |
| 4 | C.S. | 4 | Bacterial asthma | Non-hemolytic streptococcus | 14 | +++ |

* See under table 2.

hardly applicable as a letter written in April, 1923, six months after the treatment reports two slight attacks of asthma during this winter. The patient with emphysema was a doctor sixty years old whose asthma had practically incapacitated him. At present he is not "cured" but he is much better and able to carry on.

The results obtained by stock vaccines are not given in detail. The general procedure has been entirely similar to that employed in the treatment with autogenous vaccines. Three, four or more stock vaccines are selected—usually a *Staphylococcus aureus*, a mixture of different green-producing streptococci and a mixture of various hemolytic streptococci; occasionally non-hemolytic streptococci, pneumococci or *Micrococcus catarrhalis* have been used. Small test doses of each vaccine are given subcutaneously and further treatment is carried out according to the reactions obtained. Here again the success of the treatment goes hand in hand with the size of the local reactions produced. In 29 cases a local reaction to one or other stock vaccine was produced and treatment was successful in all but 1 case. Five of these patients were "cured" and 11 have been definitely benefited—a percentage of 57. While in 6 the results were only fair and in 6 others slight. In 12 cases, stock vaccines failed to produce a local reaction and there was no improvement. The 5 "cured" cases are worthy of special note:

Mrs. S., age forty-eight, had asthma for twelve years and also arthritis. A non-hemolytic streptococcus stock vaccine was given in 1920 and 1921 with considerable local reaction and for one year she has remained free of asthma and of arthritis. B. E. M., a woman of forty-three, had asthma associated with "colds" for two years, when in September, 1921, she was found to give a large local reaction to a mixed green streptococcus stock vaccine and treatment with this vaccine given by her own doctor has resulted in an apparent cure in that she has now gone for over sixteen months without asthma.

The three other "cured" cases are all children. A girl of twelve has had no asthma for over fourteen months following 6 doses of a mixed green streptococcus vaccine each dose of which produced a large local

reaction at the site of inoculation. Before the treatment, her attacks had occurred on an average of once in three weeks without relation to the seasons.

A boy of eight, subject to frequent colds aggravated by asthmatic attacks, has had no further trouble for over a year after receiving 7 doses of a stock *Staphylococcus aureus* vaccine to which he gave moderate reactions.

A child of three was entirely well for a year and a half after 5 doses of a stock *Staphylococcus albus* vaccine. At that time, however, this child had another attack following which 3 additional doses of a similar vaccine were given—the local reactions consisting of large red areas which persisted for forty-eight hours. Since this second series, he has passed a whole year with no further trouble.

Both stock vaccines and autogenous vaccines have been used in different courses in the same patient and we have observed repeatedly not only that an autogenous vaccine has helped the patient where a stock vaccine has failed: but also, and this is of greater importance although less common, that a stock vaccine has helped where an autogenous vaccine has failed. This is probably explained by the fact that the first course of treatment was not accompanied by the local reactions, which did accompany the second course.

*The type of sputum* found in asthma varies considerably. In general it is either thick and purulent or thin, watery and mucoid. Its gross appearance is of some importance as it indicates the degree of bronchial infection. In the absence of acute disease, thick purulent sputum is found most often in patients with emphysema where there probably are small areas of bronchiectasis in the lungs. The mucoid type is found in patients with simple asthma who have comparatively little bronchial infection. Between these two principal types all varieties of intermediate specimens occur. As regards any gross features of the sputum characteristic of asthma, it might be said in passing that the pearls of Laennec as well as Curschmann's spirals and Charcot-Leyden crystals were not frequently found. The finding of an increase in the eosinophile cells was, however, very common and occurred especially in mucoid specimens.

When the bacteriology of the sputum in asthma is studied and compared with the gross character, several interesting observations can be made.

TABLE 6

*Showing the numbers of sputa of both thick and thin types having the bacteriology as indicated*

| THICK PURULENT | | THIN WATERY | |
|---|---|---|---|
| Predominating organism | Other organisms* | Predominating organism | Other organisms* |
| Non-hemolytic Streptococcus. 6 | Streptococcus... 9 | Non-hemolytic streptococcus 7 | Streptococcus.. 36 |
| Green streptococcus....... 14 | Streptococcus and staphylococcus....... 9 | Green streptococcus....... 12 | Streptococcus and staphylococcus..... 12 |
| Slightly hemolytic streptococcus†....... 2 | Streptococcus and diphtheroid......... 1 | Slightly hemolytic streptococcus...... 7 | Streptococcus and large gram-negative bacilli‡..... 1 |
| Hemolytic streptococcus..... 1 | Streptococcus and influenza bacillus...... 1 | Hemolytic streptococcus 5 | |
| Pneumococcus.. 3 | | Pneumococcus. 1 | |
| Influenza bacillus.......... 10 | | Influenza bacillus.......... 2 | |
| *Staphylococcus. aureus*....... 6 | | *Staphylococcus aureus*..... 4 | |
| 42 | 20 | 38 | 49 |
| Total thick purulent sputa, 62 | | Total thin watery sputa, 87 | |
| Total sputa, 149 | | | |

* No attempt is made to differentiate the varieties of the various streptococci, and other organisms under this heading.

† Refers to a streptococcus which produces only a very narrow zone of hemolysis on a blood agar plate.

‡ See footnote ‡ under table 2.

Table 6 represents a summary of such a study. First of all predominant organisms occur in over two-thirds of thick purulent sputa whereas they occur in less than one-half of the thin sputa. Allowing for the differences in total numbers the figures given for streptococci in the two series are reasonably compar-

able although hemolytic streptococci are more frequent in thin sputum. On the other hand pneumococci, influenza bacilli and staphylococci are very definitely more common as a predominating organism in thick sputum than they are in thin sputum. In the column marked "other organisms" there are large numbers of streptococci in thin sputum. Many times it is difficult to estimate whether a certain colony is predominant or not so that the numbers given for other organisms in thin sputa are large while the number of predominant streptococci is rather small. Streptococci are more numerous and more diverse in thin sputum. The very small number of pneumococci found is due entirely to the method. It will be recalled that Stillman (7) was able to recover pneumococci from sputum by mouse inoculation in 47 instances in none of which were these organisms demonstrable by direct sputum culture on a rabbit-blood agar plate.

It is the rule to find that the character of the sputum changes with the change in the patient's condition, but it is not possible to say whether the sputum change is due to the treatment. One patient, a doctor, with a chronic bronchitis and some bronchiectasis has been followed for two and one-half years. During this period, examinations of his sputum have been made at least once in six months and occasionally at weekly intervals. When first seen, his sputum was almost pure pus and contained a type III pneumococcus in practically pure culture. He has been given a vaccine made from his own organism in large doses which have produced a moderate local reaction. He is improved, but is still subject to exacerbations which are however of short duration. During these exacerbations, his sputum shows on a rabbit-blood agar plate an almost pure culture of the type III pneumococcus, but when the attack passes off, the sputum becomes less purulent, and although the type III pneumococcus is almost always present it is mixed with various streptococci, on several occasions with influenza bacilli and recently with staphylococci. In other words prolonged vaccine treatment has failed to kill off the corresponding organism and yet the patient maintains that the treatment has improved his general

condition and has made it possible to carry on his work without the loss of time so frequent before treatment. Incidentally no agglutinins for the type III pneumococcus have been found on two occasions in his blood serum with the technic used. The sputa of other patients has likewise been examined repeatedly but with no striking results. It may be said that when the sputum becomes less purulent, the bacteriology becomes more varied and streptococci increase relatively. On several occasions different plates have been made at short intervals. The lack of close duplication is probably due to the difficulties and inaccuracies of the method. In this study the sputum has not been washed before inoculating the original plate, which may explain our discrepancies, although we find that careful selection of the proper sample together with proper emulsification on the plate gives comparable results when different plates are inoculated from the same specimen of sputum at the same time.

## DISCUSSION

There are at least three theories by which the beneficial action of vaccines in asthma may be explained. First, asthma may depend upon a low grade infection of the bronchial mucosa. This infection causes a direct local irritation of the nerve endings or perhaps of the muscles in the bronchi, or it may result in the absorption of toxins which bring about the same irritations indirectly. In either case the vaccine theoretically stimulates an active immunity toward this infection. From what we know of the lack of immune relationships between different strains of the less virulent varieties of cocci, it is clearly evident that any such action by the vaccine must be highly specific. According to a second theory, the patient may be sensitive to bacterial proteins just as other patients are sensitive to ragweed pollen or horse hair extract and the purpose of vaccine treatment is to use up the excess of antibodies in the cells and thus bring about "desensitization." Here again the action is a highly specific one. Third, vaccines produce a certain degree of "protein shock," the effect of which is never specific but is comparable

to the effect upon various chronic processes which may occur with the general reaction following the intravenous administration of typhoid bacilli, peptone, etc.

In this connection it is interesting to recall that intercurrent infections all of them of the respiratory tract, pneumonia, "grippe," tonsillitis or acute sinusitis, commonly leave an asthmatic free of his trouble for periods varying from weeks to months. We cannot recall a case of non-respiratory febrile disease in this series, but such a case would be of first importance in demonstrating whether the relief from asthma depended upon the fever, that is, on the "protein shock" or whether it depended on some local effect upon the bronchial and nasal mucous membrane and the nerve endings therein.

By reference to table 1 the total results obtained with autogenous vaccines can be compared with those obtained with stock vaccines. Ninety patients have been treated with autogenous vaccines and of these 90, 49 have been helped in some degree—a percentage of 53.

Only 41 patients have been treated with stock vaccines, but of these, 28 have been helped—a percentage of 68, which is more than 10 per cent greater than the results with autogenous vaccines. The series is rather small, but in view of these figures it may be said with some assurance that stock vaccines are at least as effective in treatment as are autogenous vaccines.

We have laid great stress upon the importance of local reactions following the injections. We are impressed that this is important and our figures justify the impression. Table 1 shows a total of 77 results which are more or less good. It is important that a total of only 4 followed treatment which did not produce a local reaction. On the other hand, a local reaction occurred in 79 cases and failed to be accompanied by a good result in only 6 cases.

There is something "peculiar" about the local reaction following the injection of vaccine. We have treated patients with various non-bacterial extracts such for example as horse hair extract, pollen extracts, dust extracts, to which they were sensitive and have produced local reactions which persisted for twenty-

four hours or more as red, swollen areas and yet which were not accompanied by any improvement in the asthma. While it is true that the vaccine reaction looks clinically much like the non-bacterial reaction, the latter produces not a cellular injury as occurs after such a non-diffusible antigen as a vaccine, but a pericellular and vascular injury as Zinsser (8) has indicated. Whether we have a right to compare these two forms of reaction is doubtful, but the difference between them is mentioned in order to bring out the importance of producing a local reaction of a certain type.

As to the mechanism of vaccine action in asthma, we can only speculate by saying that the good results with stock vaccines, the importance of using a vaccine which will produce a local reaction and perhaps the fact that in the few cases studied, no circulating antibodies have been found, the impression is that the action is non-specific.

In this report, the word "cured" has been invariably placed in quotation marks. Every effort has been made to determine the end results of all treated cases and the conclusion is forced upon us that only 9 patients in a total of 131 treated have been "cured" by vaccines. These 9 cases are of sufficient importance to warrant their tabulation (table 7).

This table shows that 4 patients were cured by autogenous vaccines and also that 5 patients were cured by stock vaccines. Although most of them have been free of asthma for periods long enough to warrant the application of the word "cured," it is important to note that at least 2 patients relapsed after a year and one-half. It is not unusual to find that adults with asthma of only a few years duration will say that as a child they did have previous asthma and often of a severe type which subsided without particular treatment.

All this is important because it indicates that even the successful treatment of a bronchial infection is not a guarantee of permanent relief from asthma. On the other hand, there is no doubt but what vaccines do good in many cases—great good in a few cases, and their use in asthma, at least in cases which cannot be treated by other and simpler measures, is reasonable

TABLE 7

*The nine "cured" cases*

| | INITIALS | AGE | DIAGNOSIS | FREQUENCY OF ATTACKS BEFORE TREATMENT (IN WEEKS) | VACCINE USED | NUMBER TREATMENTS | DATE FIRST DOSE | DATE LAST DOSE | LAST DATE KNOWN TO BE FREE OF ASTHMA |
|---|---|---|---|---|---|---|---|---|---|
| 1 | G. U. | 11 | Bacterial asthma | 4 | Autogenous non-hemolytic streptococcus | 14 | October, 1919 | May, 1920 | April, 1923 |
| 2 | D. E. | 11 | Bacterial asthma | 6 | Autogenous non-hemolytic streptococcus | 7 | December, 1919 | March, 1920 | |
| | | | | | Autogenous non-hemolytic streptococcus | 4 | May, 1922† | June, 1922 | April, 1923 |
| 3 | G. F. S. | 13 | Bacterial asthma | 2–4 | Autogenous mixed | 5 | July, 1919 | August 1919 | March, 1922 |
| 4 | S. P. | 20 | Bacterial asthma | 2–4 | Autogenous green streptococcus | 8 | November, 1922 | December, 1922 | April, 1923 |
| 5 | Mrs. S. | 48 | Bacterial asthma, Arthritis | steady | Stock non-hemolytic streptococcus | (?) | August, 1920 | January, 1921 | November, 1922 |
| 6 | B. E. M. | 43 | Bacterial asthma | 2–6 | Stock mixed green streptococcus | (?) | September, 1921 | (?) | March, 1923 |
| 7 | M. N. | 12 | Bacterial asthma | 3 | Stock mixed green streptococcus | 6 | August, 1921 | December, 1921 | March, 1923 |
| 8 | O. B. | 8 | Bacterial asthma | 2–4 | Stock *Staphylococcus aureus* | 7 | November, 1921 | December 1921 | January, 1923 |
| 9 | R. M. | 3 | Bacterial asthma | 6–8 | Stock *Staphylococcus albus* | 5 | October, 1920 | November, 1920 | |
| | | | | | Stock *Staphylococcus albus** | 3 | March, 1922† | March, 1922† | April, 1923 |

* A different vaccine was used for the second course of treatment.

† Relapse but well in the interval.

and justifiable. Until we can discover the true pathological physiology of asthma it will be quite impossible to define the indications for the use of vaccines and until we understand the mode of action of vaccines, it will be quite impossible to use them with accuracy.

### SUMMARY

1. The method of making autogenous vaccines and of treating patients with emphysema, chronic bronchitis and with bacterial asthma is described.

2. Ninety patients have been treated with autogenous vaccines. A predominating organism was found and used in 29 cases with good results in 11 and fair results in 11. In 61 cases, there was no predominating organism but a selection from several autogenous vaccines was made on the basis of the local reactions following the test dose. Good results occurred in 13 and fair results in 14.

3. Forty-one patients have been treated with stock vaccines likewise selected on the basis of the local reactions with good results in 16 and fair results in 12.

4. Whatever vaccine is used the fact that good results occur only in those cases where the various doses of the vaccine are followed by local reactions at the site of inoculation is definite and striking.

5. The importance of the local reaction plus the fact that the results with stock vaccines are as good or better than those with autogenous vaccines gives rise to the impression that the action of all vaccines in asthma is non-specific.

6. Of the entire series of 131 patients 9 or 7 per cent have been "cured;" 31 or 22 per cent have been definitely benefited; 13 or 10 per cent have been helped somewhat; 24 or 18.5 per cent have been relieved temporarily but 54 or 41 per cent have not been relieved at all.

7. Although good proof of a cure brought about by vaccines is lacking, vaccines do good in many cases. And at least until we know more of the fundamental pathological physiology of asthma, their use is reasonable and justifiable.

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# SEROLOGICAL REACTIONS IN ISOLATED RABBIT LUNGS

## I. REACTIONS TO HISTAMINE AND TO VAUGHAN'S PROTEIN SPLIT PRODUCT

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It was shown by Coca (1) that the dominant physiological reaction in acute anaphylaxis in rabbits is an occlusion of the pulmonary blood vessels with red blood corpuscle masses. Coca believes this occlusion is not due to thrombosis or hemagglutination (embolism), but that it is secondary to an acute pulmonary vasoconstriction comparable with the acute bronchoconstriction in guinea pigs. As a preliminary to a serological analysis of this reaction, we have studied the reactions of isolated rabbit lungs to histamine and to Vaughan's protein split product.

Our tests were made by perfusion methods. To make the pulmonary perfusion, the ascending aorta is ligated, an afferent cannula is tied in the pulmonary artery, and an efferent cannula in the left auricular appendage. The lungs are then inflated to their midespiratory volume and the trachea clamped. The perfusion fluid is well-aerated Locke's solution, at 38°C. Perfusion pressure, 11 to 13 mm. Hg.

### HISTAMINE REACTIONS

On histamine perfusion of normal rabbit lungs there is a very marked increase in the perfusion resistance, the rate of perfusion flow being reduced fully 95 per cent. With concentrated histamine solutions (fig. 1, *A*) this vasoconstriction reaches its maximum within sixty seconds. With dilute histamine solutions

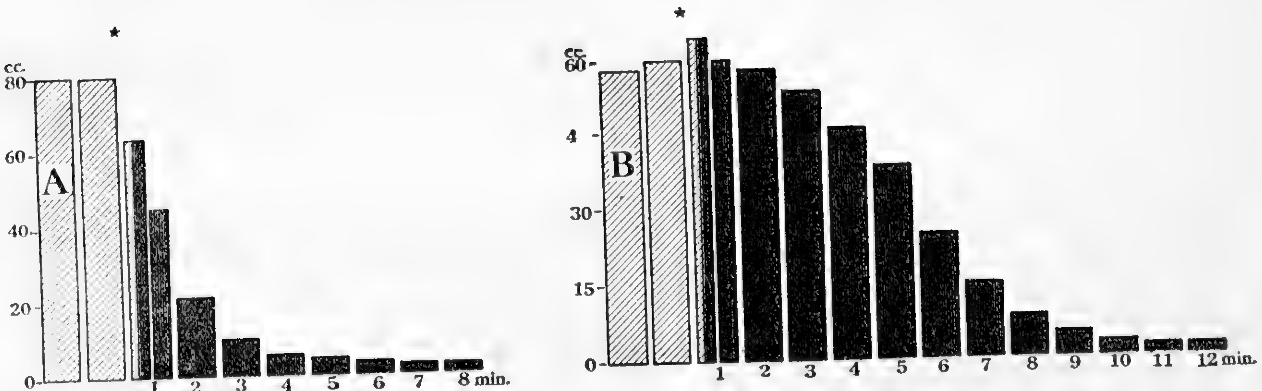


FIG. 1. HISTAMINE REACTIONS IN NORMAL RABBIT LUNGS

Cross-hatched areas show rate of perfusion flow per minute with Locke's solution. Black areas show rate per minute (or half-minute) with Locke's solution plus histamine. Star (*) shows time of changing the perfusion clamps.

*A* = Perfusion with 1 mgm. histamine per 100 cc. Locke's solution.
*B* = Perfusion with 0.25 mgm. histamine per 100 cc. Locke's solution.

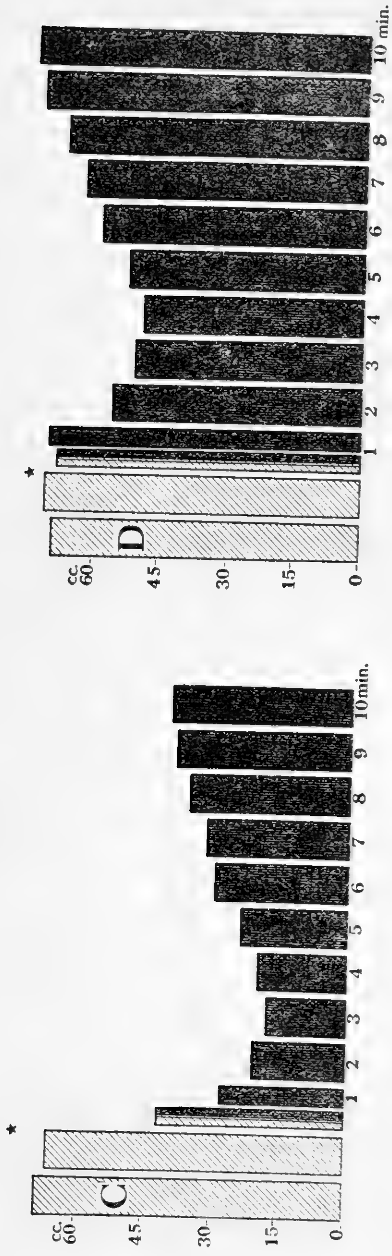


FIG. 2. REACTIONS OF NORMAL RABBIT LUNGS TO VAUGHAN'S PROTEIN SPLIT PRODUCT

Cross-hatched areas show perfusion flow per minute with Locke's solution. Black areas show rate per minute (or half-minute) with non-alkaline Locke's solution plus Vaughan's protein split product.

*C* = Perfusion with 40 mgm. split product per 100 cc. Locke's solution.
*D* = Perfusion with 10 mgm. split product per 100 cc. Locke's solution.

(fig. 2, *B*), the vasoconstriction is preceded by a slight preliminary vasodilation, and does not reach its maximum till the sixth to tenth minute. No tendency to recovery was noted in any of our tests.

If at the end of the perfusion the tracheal clamp is released, the lungs collapse normally. No frothy fluid escapes from the trachea. The only change noted on gross section of the lungs is a marked perivascular edema, similar to the perivascular edema previously described for the isolated canine lungs (2), each blood vessel being surrounded by a well-marked edematous zone. Whether or not the increased perfusion resistance is due wholly to a passive narrowing of the larger blood vessels as a result of increased perivascular pressure, cannot be determined from our present data. A histological study of the factors entering into the reaction will be reported later.

## REACTIONS TO VAUGHAN'S PROTEIN SPLIT PRODUCT

The split product used in these tests was obtained for Dr. Vaughan's laboratory. It is so toxic that 1 mgm. injected intravenously will kill a medium-sized guinea pig in from three to five minutes with characteristic symptoms of acute anaphylaxis. On perfusing the isolated rabbit lungs with this product dissolved in nonalkaline Locke's solution, there is a marked increase in perfusion resistance, the perfusion rate being reduced from 25 to 75 per cent within three minutes, depending upon the concentration of the product used. Recovery begins during the fourth minute. With larger doses of the split product (fig. 2, *A*), the recovery is only partial. With smaller doses (fig. 2, *B*), the recovery is complete by the end of nine minutes.

If at the end of the perfusion the tracheal clamp is released, the lungs collapse normally. No frothy fluid escapes from the trachea. On gross section of the lungs, a distinct perivascular edema is noted.

## SUMMARY

1. A vasoconstriction sufficient to reduce the rate of perfusion flow fully 95 per cent is produced by histamine in the isolated rabbit lungs. This vasoconstriction is associated with a marked perivascular edema.

2. A 75 per cent reduction in perfusion flow is produced by Vaughan's protein split product, with partial or complete recovery by the end of nine minutes.

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# STUDIES IN ENDOTHELIAL PERMEABILITY. I

## THE EFFECT OF EPINEPHRIN ON ENDOTHELIAL PERMEABILITY

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### INTRODUCTION

In the following series of papers we describe experiments that deal primarily with observations on endothelial permeability as studied by means of lymph analysis. The number of theoretical as well as practical therapeutic problems that are related in an intimate way to alterations of endothelial permeability may justify a study such as this even though we already possess a great amount of information gathered in the laboratories of Ludwig, Heidenhain, Asher, Carlson, Starling and others. Much of the older work was carried out at a time when the knowledge concerning the physico-chemical factors that might modify the permeability of the endothelium had not been developed, while more gross forces, as osmosis, were perhaps overemphasized. The more recent communications have been quite numerous and in them cognizance has been taken of the modern concepts concerning cellular structure. In these the earlier leads of Asher and Starling have been developed and the work of Meyer-Bisch (1), Bauer and Ashner (2), and Osato (3) begins to throw a new light on some of the findings of earlier workers which had been obscure. On examination of these recent contributions, most of which have been made by physiologists, one finds practically no mention of the pathologic significance of the observations (except the discussion of edema, the relation of which is obvious), and excepting the paper by Meyer-Bisch, no consideration of the therapeutic questions involved. It is this latter analysis which we hope to present in these papers.

Ebbecks (4) recently pointed out that stimulation of cells, increase in permeability and an increase in electrical conductivity parallel each other. As a matter of fact the increase in permeability is a symptom, an indicator of excitation. Excitation produced by any irritant (Reiz) is accompanied by increased oxygen consumption and carbon dioxide production, increased production of lactic and phosphoric acid, the setting up of action currents as well as the more obvious changes such as motion, secretion, sensory changes, etc. The converse that rest, lessened permeability, and static electrical conditions go hand in hand seems also well established. The impermeability of the membrane prevents a loss of soluble constituents and permits the association of the cell specific electrolytes.

A number of investigators have taken part in establishing this view. Thus Lilly (5) as a result of his work postulates that stimulation is associated with a reversible increase in cell permeability, an increase that is due to an alteration (or temporary breakdown) of the protoplasmic film structure of the limiting surface membranes. Hoeber (6) Gildermeister (7), Bernstein (8), Hermann (9), Vorschutz (10), Osterhaut (11), and Embden and his school (12) have shared in this work. Lilly in particular emphasizes the importance in the bioelectrical changes that initiate the intermittant and reversible changes in the cell membranes. Certainly this is not the relatively static and fixed structures the older physiologists conceived but functionally an exceedingly labile structure that is of paramount importance in influencing the whole activity of the cell. We may consider that the highly reactive tissues such as nerve and muscle and special sense organs are such by virtue of the great lability of their surface membranes, while the cells of the inert supporting structure, bone, connective tissue, etc., might be considered as occupying the opposite end of the scale. In the intermediate group we may be justified in grouping endothelium, glandular parenchyma, leukocytes, slowly contracting muscle (Behrendt, 13), etc.

While greatest emphasis has been laid in these various studies on the effect of physiological stimulation and the associated increase in permeability, the fact that the cell continues to become more and more permeable when continuously stimulated (Embden) must be remembered. When the stimulus is a continuous one that lies below the threshold of an obvious induction of a reaction (Weiss (14) used subthreshold stimulation of muscle cells) this increase in permeability can be readily observed. The evident bearing of this observation on a view expressed by Hermann that there is no clear line of demarcation between rest and irritation and between irritation and inflammation may be of interest. According to Hermann we should speak of degrees of functional and metabolic activity.

The fact that the cell when at rest is relatively impermeable deserves equal attention. If the stimulated cell becomes more susceptible to a following irritation; i.e., is more labile than the normal resting cell, and more readily fatigued, as suggested by Hermann, we might suppose that when the reverse of stimulation, the state of rest exists, a difference in the degree of resistance to change (a tendency to maintain the status quo) may be manifest. As a result of a previous stimulation of extraordinary magnitude the static phase might become more pronounced.

We have dwelt at some length on these more or less general conceptions without entering into a discussion of the theories and observations that deal with the possible structure of the surface membranes because the membrane changes that occur with cell stimulation have a direct bearing on the problem under consideration. The endothelial cell does not differ in its fundamental reactions from any other cell. Stimulation must be associated with increased permeability, fatigue with its continuation and death with complete permeability. Rest, if absolute, is associated with impermeability. Reasoning on the basis of anatomical structure and obvious function we may assume that the endothelium has become specialized in the control of permeability.

We should like to mention at this place that it seems probable that the state of rest and impermeability practically does not exist in endothelium when the capillary is open. It must normally be under a constant stimulus. If not mechanical, due to the movement of the fluids in contact with it, then chemical, as a result of alteration in reaction and the presence of tissue metabolites from alterations in the activity of the adjacent cells. While, therefore, theoretical impermeability may exist, actually the endothelium is permeable for water, crystalloids and colloids to a variable degree. In this mechanism of permeability osmosis, diffusion, ultrafiltration, colloidal hydrations and dehydration incident to alterations in reaction, all have their share and must be considered. It seems most probable that the actual changes in permeability would depend on this latter factor, that is, on a change in the hydration or the dehydration of the colloids making up the surface film. This in its turn would modify the behavior of the surface film as an ultrafilter.

We must turn for a moment to examine the relative size of the particles that as hydrated colloids make up the serum plasma. It has been determined that these range themselves in the following order of magnitude: fibrinogen, euglobulin, pseudoglobulin I, pseudoglobulin II, albumin, albumoses. According to the current conception these exist as fairly constant entities in the blood plasma, more or less distinct one from the other. Within the past few years, however, Herzfeld (15) and Hirschfeld and Klinger (16) have suggested on the basis of quite interesting experiments that what we are really dealing with is an unbroken chain of molecular aggregates diminishing in size from fibrinogen to the amino acids. According to their conception the serum proteins are not formed as a distinct secretion from any one group of cells but represent instead the proteins liberated by the general breakdown of tissue cells. When this takes place the largest fragments form fibrinogen. This is kept in unstable solution by the adsorption on the surface of the aggregate of a large number of smaller colloidal particles; any process that diverts these results in the precipitation of the fibrinogen in an insoluble form. Any agent that increases the adsorption of

these smaller aggregates on the surface, or any agent that enzymatically breaks down the fibrinogen aggregates (as trypsin, peptone, etc.) would of course render the plasma incoagulable. This theory is attractive in many ways, but a number of objections can be brought against it, particularly regarding the fibrinogen. In general we believe that the conception that the plasma proteins form a continuous and unbroken chain of molecular aggregates of increasing diminution in size is one that has much to commend it.

If we consider the passage of colloids through the endothelial membrane to the lymph stream in the nature of an ultrafiltration (and leave out of consideration for the time being the alterations that may depend on osmosis, diffusion, hydration and dehydration) then the exchange will depend on two factors, pressure and size of filter openings. In the stimulated cell, as we have shown, we have every reason to assume that the size of the filter openings is increased, in the resting cell, diminished. We might expect some differential filtration of colloids under these conditions that will vary with the state of activity of the cell. Filtration must be considered as an expression of a cellular function in which the state of activity or rest of the cell plays a vital rôle.

When we examine the lymph flowing from the thoracic duct we must keep in mind that other factors than these may modify its composition. We may expect that the differences in the amount of protein in the lymph from the extremities and from the abdominal area will be such a factor if at any time the amount of one is increased or diminished (that from the extremeties being as a rule considerably poorer in total solids). The amount of water may be modified after the capillary endothelium has been passed because of alterations in the reaction of the tissue through which it later passes. The amount of solids may finally be increased considerably if a process of cellular disintegration is going on. Keeping these possibilities in mind we have sought to determine whether or not a differential filtration of lymph can be determined and in what measure it may be modified by a number of procedures.

## METHOD

We have used dogs exclusively in this work. We have selected medium sized animals (from 7 to 12 kilos as a rule), discarding irritable or nervous animals. We have avoided general anesthetics because of the marked effects which these have on the permeability of cells: thoracic duct cannulas were inserted under local anesthetic. If the animals were restless for any considerable period after the operation, they were discarded. As a rule they were kept under observation on the board from two to six hours and the lymph was collected continuously, samples being taken for ten or fifteen minute periods and when the flow was relatively small, pooled samples were used for analysis.

*Differential estimation of proteins.* The fibrin was collected, dried and weighed (Gram method) (17). Kjeldahl analysis was made for total nitrogen of the lymph. The globulins were estimated turbidimetrically as follows: The concentrations of sodium sulphate recommended by Howe (17) were used and the following tubes set up:

0.5 cc. of lymph with 15 cc. of physiological salt solution (for turbidity control)
0.5 cc. of lymph to which was added 15 cc. of a 14 per cent sodium sulphate
0.5 cc. of lymph to which was added 15 cc. of an 18 per cent sodium sulphate
0.5 cc. of lymph to which was added 15 cc. of an 22.2 per cent sodium sulphate
0.05 cc. of lymph was precipitated with sulphosalicylic acid according to the method described for the estimation of protein in urine by Folin and Denis (19). This latter was used as a check on our Kjeldahl determinations.

The precipitates were all read in the Kober colorimeter (using it as a turbidimeter) against appropriate standards made with a solution of serum so diluted that 1 cc. contained 5 mgm. of protein, and precipitated with sulphosalicylic acid. We are aware that the values obtained by this method are by no means absolute, but the comparative values for a series of observations

made under the same conditions of precipitation, etc., are good. Micronitrogen determinations in the very large number called for in these examinations would be practically out of the question and we doubt whether the range of possible error might not be just as great. In making the estimations the control of the inherent turbidity must of course be deducted. In order to reduce this to the lowest amount the animals were usually given no food for 36 hours before the experiment. The difference between the Kjeldahl titration estimated as protein ($\times$ 6.5) and the globulins plus fibrin has been tabulated under the heading "albumin." This includes the non-colloidal nitrogen; all amounts have been calculated in per cent of the total protein (Kjeldahl $\times$ 6.5 plus fibrin).

*Permeability to hemoglobin.* As an additional check on this method we have used hemoglobin to determine the permeability of the endothelium for colloids. In many ways this offers an ideal method because we are dealing with a substance which is not foreign as are all other dyes that have been suggested. Osato has for instance, made use of sulphophenolphthalein and indigo carmine; both of these dyes pass through very rapidly (in from four to five minutes) and promptly reach a maximum concentration. These dyes have the disadvantage that as foreign substances they may in themselves stimulate the endothelium. The size of the hemoglobin aggregate, according to Bechhold (20) is somewhat larger than that of serum albumin and he makes use of hemoglobin to test the permeability of ultrafilter membranes for colloids. The passage of hemoglobin should therefore afford us additional evidence of the approximate rate with which colloids can pass the endothelial membranes, if they are of approximately the same size. Naturally the rate of destruction of the hemoglobin after injection into the blood stream will be a variable factor in different animals.

Our solution of hemoglobin was prepared as follows: Dog corpuscles were washed, concentrated and laked with a minimal amount of ether. Two volumes of aluminum cream (21) were added to the mass to precipitate the proteins and the mixture filtered. The clear solution of hemoglobin was then made

isotonic, warmed, and an air current passed through the solution until all ether was removed. When slowly injected (five minutes) and warmed to body temperature we find that we can inject 20 cc. of such a solution intravenously without altering the rate of the lymph flow and evidently without primary toxic effect. In some instances we have observed a chill in the animals in from thirty to sixty minutes after injection, perhaps due to a dissociation of the hemoglobin and the liberation of globin. This Weichardt and Schittenhelm found to be toxic.

Normally the pigment makes its appearance in the lymph in from twelve to fifteen minutes, reaching a maximum rather slowly (from one to two hours). The relative amount present in the plasma or lymph can be determined, colorimetrically quite accurately when the lymph or plasma is clear.

## THE EFFECT OF EPINEPHRIN ON ENDOTHELIAL PERMEABILITY

Among the effects of epinephrin on the organism one of considerable therapeutic importance, namely, the possibility of altering cell permeability, has been the subject of controversy since Asher (22) and his pupil Böhm (23) published experiments which showed that an increase in capillary pressure was not necessarily followed by an increased filtration of fluids through the capillary wall. Exner (24) and later Meltzer and Auer (25) found that epinephrin diminished the adsorption of fluids from the peritoneal cavity. Hess (26) and Erb (27) approached the problem from a different position. They both found that the blood concentration increased after the injection of epinephrin; one determined this by the corpuscular count, the other by weighing the solids of the blood. This alteration they assumed was due to an increased filtration of fluids from the blood stream into the tissue spaces and lymph channels. It would be reasonable to suppose that with the return to a normal blood pressure the primary alteration would be rapidly compensated by a reverse in the current. As a matter of fact it was found that the increase in concentration persisted for a much longer period

of time than could be accounted for on this basis and both Hess and Erb reached the conclusion that some alteration in permeability must have taken place. Asher and Böhm confirmed the findings of Hess and Erb but did not make the same interpretation. They were able to demonstrate that with increased capillary pressure no increase in filtration need follow; on the other hand with vasodilation and lowering of capillary pressure (as shown by Dale, 28) we may have a marked increase in permeability. Vasoconstriction and dilatation on the one hand and increased or diminished permeability on the other are not necessarily related. The work of Asher and his associates was passed over by several workers, among them Bertelli, Falta and Schwerger (29), Donath (30), Schenk (31), Bilingheimer (32), Full (33) and others, who explained their results on the basis that the increase in arterial and presumably, capillary pressure, increased the filtration and that later the capillaries became impermeable. Two observers worked with thoracic duct fistulas; Camus (34) found that the lymph flow was increased after epinephrin injections, Thomaszewski and Wilenko (35) found it diminished.

Bauer and Ashner (36) in studying the concentration of the blood of patients after epinephrin injections found that in some instances the concentration was diminished, in others increased. They have come to the conclusion that the increased blood pressure does not account for the concentration of the blood, because in some of their cases they were able to determine such an increase when no increase in blood pressure was observed. Nor did they agree that the continued increase in concentration, when it followed injections, was due to alteration of permeability, because they suppose that this should cease when the pressor effect of the epinephrin had ceased.

There are however certain other observations that have some bearing on the subject. Jona (37), Clark (38) and Cobet and Ganter (39) have studied the effect on the absorption of exudates (peritoneal and pleural) while Athanasiu and Gradinescu (40) have made observations on the influence of epinephrin on the swelling of excised frog muscle. The epinephrin prevents

this to a considerable extent, as Donath (41) found in similar studies on the excised cat kidney. More recently Langer (42) has presented evidence from Embden's laboratory which proves that epinephrin is able to diminish the permeability of frog muscle. This he did by studying the diffusion of phosphoric acid from the excised muscle.

Dorothy Hutchinson (43) has found that concentrated solutions of epinephrin diminish the carbon dioxide output of muscle (and radish seedlings) and it has been previously reported that the injection of epinephrin is followed by a reduction in the metabolism of the liver.

## EXPERIMENTAL

In order to determine whether the two methods that we have outlined at the beginning of this paper might enable us to arrive at some definite answer to this particular problem we have studied the effect of intravenous injections of epinephrin on the lymph flow of dogs. The following protocols will illustrate our results.

June 27, 1922. Dog 380. Weight 8 kilos. *Effect of repeated large doses:* This animal was injected with 3 doses intravenously each of 10 cc. (1:10,000 dilution of epinephrin). The effect of the injections on the rate of lymph flow is of interest (chart 1). It will be observed that each injection is followed by a distinct increase in the lymph flow, this followed by a diminution in the flow below the preinjection level. The second and third injections result in a lessened reaction, which, while each time as great proportionally, actually diminishes in amount.

From table 1 it will be observed that the first injection was followed by the appearance of red blood corpuscles in the lymph; the succeeding injections did not have this effect. Each injection was followed by the reduction in the concentration of proteins in the lymph with a gradual return to the preinjection figures during the course of the next hour. It will be observed that the original level is never again reached and after the third injection is approximately 20 per cent less than at the beginning of the experiment. This, in conjunction with the lessened

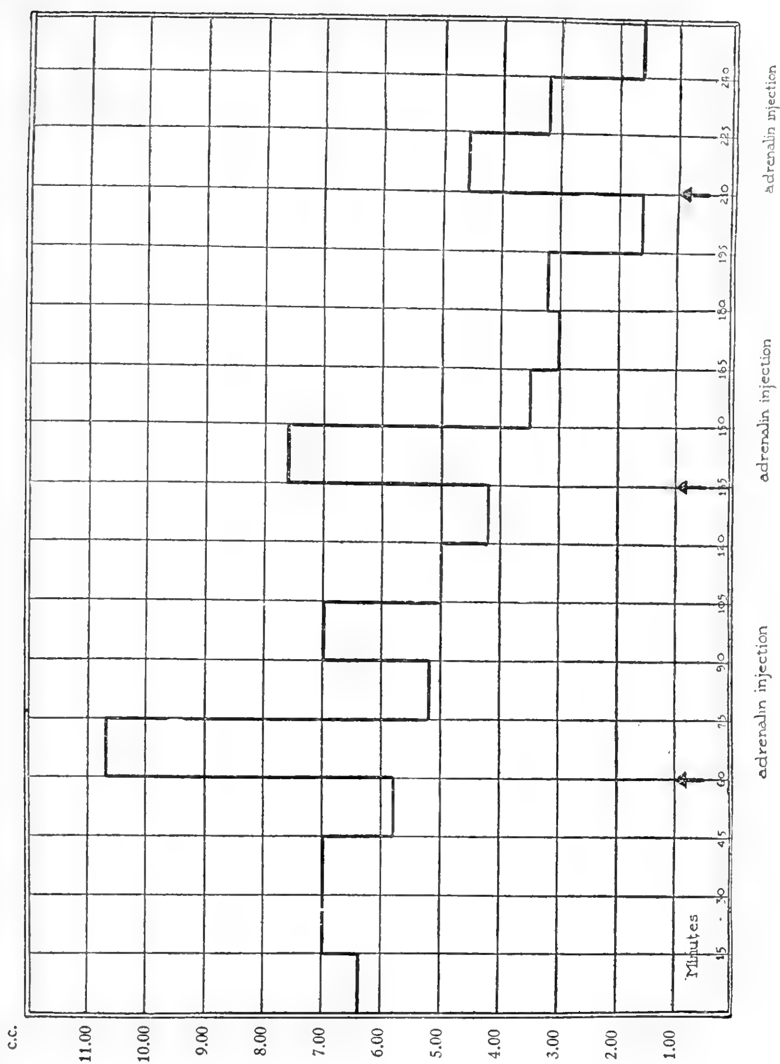


CHART 1. DOG 380. EFFECT OF REPEATED INJECTIONS OF ADRENALIN ON THE LYMPH FLOW

flow would indicate a reduction in permeability. Immediately after each injection it will be observed that the globulins are relatively increased (column 9). This can probably be interpreted as due to an increase in permeability for a short period of time following the injection or to a transient stimulation of the liver parenchyma. Fibrinogen was increased immediately, but reached a maximum at a period when the maximum vascular

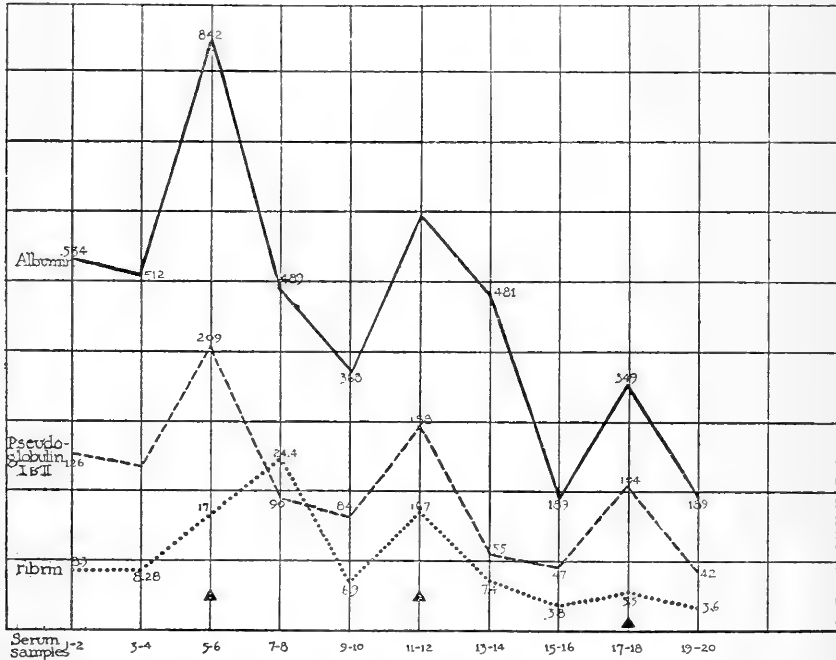


CHART 2. DOG 380. CHART OF VOLUME-PER CENT OF LYMPH PROTEINS FOLLOWING REPEATED INJECTIONS OF ADRENALIN

effect of the injection had passed. This is brought out in chart 2 in which the percentage of the different lymph constituents has been multiplied by the actual volume. From the chart the lag of the fibrinogen is quite apparent. This we believe can be explained only on the basis that the fibrinogen comes principally from the liver and that the effect of the liver stimulation and fibrinogen mobilization comes after the endothelial effect. After

TABLE 1

*Protocol of lymph analysis after repeated injection of adrenalin*

Dog 380. June 27, 1922

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NUMBER | TIME | VOLUME | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | PSEUDOGLOBULINS I AND II | ALBUMIN |
| | | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 1 | 10:00–10:15 | 6.4 | 39.9 | 1.25 | 0 | 16.5 | 2.5 | 19 | 79.75 |
| 2 | 10:15–10:30 | 7.0 | | | | | | | |
| 3 | 10:30–10:45 | 7.0 | 41.8 | 1.3 | 0 | 17.1 | 1.5 | 18.6 | 80.0 |
| 4 | 10:45–11:00 | 5.8 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 5 | 11:00–11:07 | 2.0 } 10.7 | 33.56 | 1.6 | 0 | 18.5 | 1.2 | 19.7 | 78.7 |
| 6 | 11:07–11:15 | 8.7* | | | | | | | |
| 7 | 11:15–11:30 | 5.2* | 37.0 | 4.0 | 0 | 14.1 | 1.7 | 15.8 | 80.2 |
| 8 | 11:30–11:45 | 7.0† | | | | | | | |
| 9 | 11:45–12:00 | 5.0 | 39.6 | 1.5 | 0 | 14.4 | 4.0 | 18.4 | 80.1 |
| 10 | 12:00–12:15 | 4.2 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 11 | 12:15–12:22 | 1.0 } 7.6 | 31.9 | 2.2 | 0 | 16.0 | 3.6 | 19.6 | 78.2 |
| 12 | 12:22–12:30 | 6.6 | | | | | | | |
| 13 | 12:30–12:45 | 3.5 | 34.6 | 1.7 | 0 | 14.8 | 1.7 | 16.5 | 81.8 |
| 14 | 12:45– 1:00 | 3.0 | | | | | | | |
| 15 | 1:00– 1:15 | 3.2 | 37.6 | 1.6 | 0 | 16.3 | 3.0 | 19.3 | 79.1 |
| 16 | 1:15– 1:30 | 1.6 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 17 | 1:30– 1:37 | 0.6 } 4.6 | 31.8 | 1.2 | 0 | 18.0 | 4.8 | 22.8 | 76.0 |
| 18 | 1:37– 1:45 | 4.0 | | | | | | | |
| 19 | 1:45– 2:00 | 3.2 | 33.5 | 1.5 | 0 | 16.7 | 1.2 | 17.9 | 80.8 |
| 20 | 2:00– 2:15 | 1.6 | | | | | | | |

* Red blood corpuscles in the lymph.

† Dog restless.

TABLE 2

*Protocol of lymph analysis after repeated injections of adrenalin*

Dog 378. June 26, 1922

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NUMBER | TIME | VOLUME | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | PSEUDOGLOBULINS I AND II | ALBUMIN |
| | | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 1 | 11:00–11:15 | 11.0 | 31.4 | 3.1 | 0 | 25.6 | 18.1 | 43.7 | 53.2 |
| 2 | 11:15–11:30 | 7.7 | 28.9 | 3.1 | 0.7 | 20.2 | 15.0 | 35.2 | 61.0 |
| 3 | 11:30–11:45 | 7.9 | | | | | | | |
| 4 | 11:45–12:00 | 7.85 | 31.7 | 3.1 | 1.0 | 21.1 | 14.8 | 35.9 | 60.0 |
| 5 | 12:00–12:15 | 7.83 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 6 | 12:15–12:22 | 6.5 } 11.5 | 31.7 | 3.2 | 2.0 | 19.0 | 17.3 | 36.3 | 58.5 |
| 7 | 12:22–12:30 | 5.0 | | | | | | | |
| 8 | 12:30–12:45 | 9.0 | 27.5 | 3.6 | 0.5 | 15.3 | 7.0 | 22.3 | 73.6 |
| 9 | 12:45– 1:00 | 8.4 | | | | | | | |
| 10 | 1:00– 1:15 | 5.6 | 22.8 | 3.9 | 0.6 | 31.1 | 0 | 31.1 | 54.4 |
| 11 | 1:15– 1:30 | 5.2 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 12 | 1:30– 1:37 | 4.0 } 7.7 | 30.7 | 3.0 | 1.0 | 21.8 | 14.0 | 35.8 | 60.2 |
| 13 | 1:37– 1:45 | 3.7 | | | | | | | |
| 14 | 1:45– 2:00 | 7.3 | 24.8 | 3.4 | 1.0 | 23.8 | 8.0 | 31.1 | 64.5 |
| 15 | 2:00– 2:15 | 5.1 | | | | | | | |
| 16 | 2:15– 2:30 | 6.5 | 25.3 | 5.5 | 1.4 | 21.9 | 3.5 | 25.4 | 67.7 |
| 17 | 2:30– 2:45 | 6.4 | | | | | | | |
| 10 cc. adrenalin 1:10,000 intravenously | | | | | | | | | |
| 18 | 2:45– 2:52 | 3.85 } 8.05 | 26.4 | 4.5 | 0.5 | 20.2 | 11.0 | 31.2 | 63.8 |
| 19 | 2:52– 3:00 | 4.2 | | | | | | | |
| 20 | 3:00– 3:15 | 8.8 | 23.18 | 3.7 | 0.2 | 18.6 | 10.5 | 29.1 | 67.0 |
| 21 | 3:15– 3:30 | 6.5 | | | | | | | |
| 22 | 3:30– 3:45 | 5.8 | 24.0 | 2.6 | 0.4 | 14.6 | 6.5 | 21.1 | 75.9 |
| 23 | 3:45– 4:00 | 4.4 | | | | | | | |

the third injection it will be noted that the amount of fibrinogen mobilized is greatly diminished. That an increase in fibrinogen takes place in the human after epinephrin injections has been observed by Vanysek (44).

*Conclusion.* The experiment indicates that with each injection there results a primary transient increase in permeability (stimulation) as evidenced by the increased flow and the temporary increase in the proportion of globulins. This is followed by a period of diminished permeability that becomes more manifest with each succeeding injection.

June 26, 1922. Dog 378. Weight 8 kilos. *Effect of repeated large doses:* Injections of epinephrin similar to the first animal. It will be observed from chart 3 in which the lymph flow has been charted, that the experiment is similar to the first in that there is a final reduction in the amount of the flow and that the second injection produces less increase than the first injection. Here, too, protocol 2 demonstrates the ultimate diminution in the total protein contained in the lymph, but the experiment differs from the preceding in that each injection is followed (except after the first injection) by an increase in the protein immediately after the injection. This, together with the more apparent increase in the proportion of the globulins (I and II) to the albumin leads us to believe that in this animal the effect of the primary stimulation is more apparent. Following this primary alteration the reversal of permeability becomes apparent in the diminution of protein and the relative increase in albumin and smaller molecular aggregates. The fibrinogen, as in the previous experiment, shows an independence that becomes apparent in chart 4 in which the volume per cent has been charted.

*Conclusions.* The experiment illustrates, perhaps more clearly than the previous one, that we deal with a transient primary stimulation of the endothelium (indicated by increased flow, increase in total protein and in the relative amount of globulin); this to be followed by a reversal of the conditions with lessened protein and relatively more albumin. The fibrin again takes an independent course that would indicate a delayed liver stimulation, here again the third injection no longer produces an apparent effect. This might be associated with an exhaustion of available fibrinogen or the relative immunity of the liver

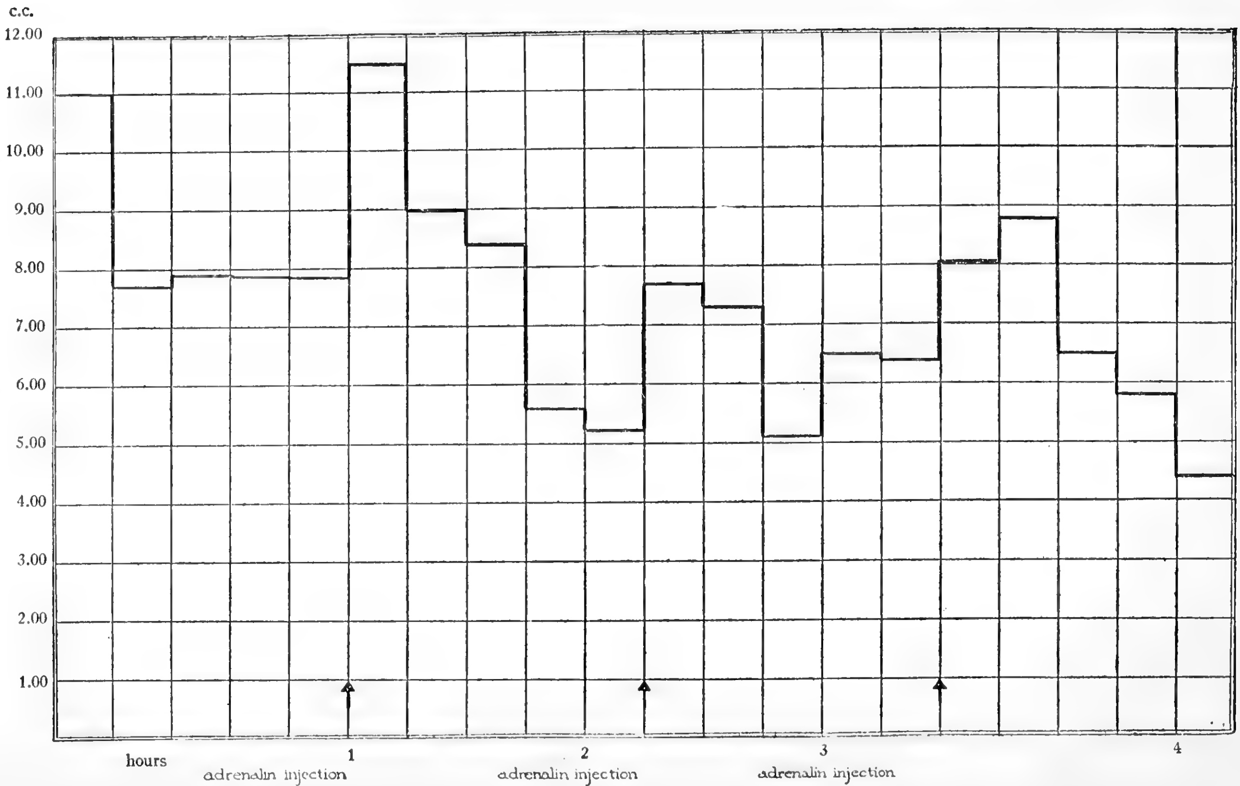


CHART 3. DOG 378. EFFECT OF REPEATED INJECTIONS OF ADRENALIN ON THE LYMPH FLOW

to further stimulation because of the change in the endothelium, which may in this case act as a buffer.

In the following experiment we present the results obtained with a *very small dose* of epinephrin.

Dog 419. Weight 8.5 kilos. Two injections, each of 1:100,000 epinephrin, the first 1 cc. intravenously. It will be observed from chart 5 and protocol 3 that the first injection produced only a slight increase in lymph flow (30 per cent) and this was not immediate but about one-half hour after the injection. After the second dose there

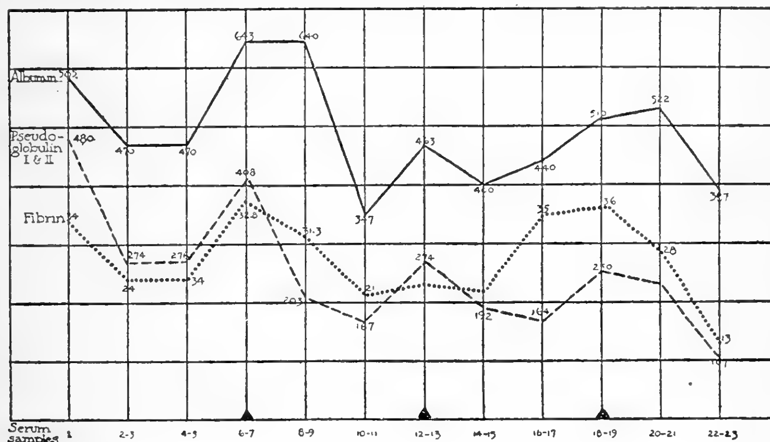


CHART 4. DOG 378. CHART OF VOLUME-PERCENT OF LYMPH PROTEINS FOLLOWING REPEATED INJECTIONS OF ADRENALIN

was first a slight diminution and then an increase a half hour after the injection. With these small doses the effect may then be a primary diminution, as has occasionally been observed by other investigators. The amount of protein increased slightly after the first injection but after the second injection were considerably diminished. This reduction is evident too, in the amount of globulin which comes through after the second injection. The albumin would be relatively increased. The fibrinogen curve was not greatly altered.

The experiment makes probable that when dealing with these smaller doses of adrenalin the primary stimulation of endothelium seems negligible but the effect in rendering the wall less permeable (relatively more albumin filtered) is still evident. The lack of stimulation seems evident in the constancy of the fibrinogen curve, which would indicate that the liver had taken little or no part in the reaction.

TABLE 3

*Protocol of lymph analysis after small dose of adrenalin*

Dog 419

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NUMBER | TIME | VOLUME | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | TOTAL GLOBULIN | ALBUMIN |
| | | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 1<br>2 | 10:00–10:15<br>10:15–10:30 | 4.9<br>3.95 | 39.9 | 4.7 | 1.0 | 2.3 | 1.42 | 17.5 | 77.8 |
| 3<br>4 | 10:30–10:45<br>10:45–11:00 | 3.9<br>3.6 | 34.8 | 4.8 | 2.0 | 3.1 | 17.4 | 22.6 | 72.7 |
| 5<br>6 | 11:00–11:15<br>11:15–11:30 | 3.5<br>2.0 | 30.9 | 4.2 | 1.0 | 1.3 | 14.6 | 16.9 | 78.9 |
| Adrenalin 1 cc. 1:100,000 intravenously | | | | | | | | | |
| 7<br>8 | 11:30–11:45<br>11:45–12:00 | 2.15<br>2.25 | 30.5 | 3.0 | 0.6 | 1.5 | 13.3 | 15.4 | 81.8 |
| 9<br>10 | 12:00–12:15<br>12:15–12:30 | 2.8<br>2.3 | 32.2 | 4.1 | 0.9 | 2.0 | 14.7 | 17.6 | 78.3 |
| Adrenalin 2 cc. 1:100,000 intravenously | | | | | | | | | |
| 11<br>12 | 12:30–12:45<br>12:45– 1:00 | 1.65<br>2.55 | 31.4 | 4.4 | 0.5 | 1.2 | 12.4 | 14.1 | 81.5 |
| 13<br>14 | 1:00– 1:15<br>1:15– 1:30 | 3.4<br>2.15 | 27.8 | 4.2 | 0.3 | 1.6 | 12.0 | 13.9 | 81.9 |
| 15<br>16 | 1:30– 1:45<br>1:45– 2:00 | 2.8*<br>3.15* | 26.76 | 4.3 | 0.6 | 1.1 | 10.8 | 12.5 | 83.2 |
| 17<br>18 | 2:00– 2:15<br>2:15– 2:30 | 3.05*<br>2.5* | 25.2 | 4.7 | 0.4 | 1.1 | 11.1 | 12.6 | 82.7 |
| 19<br>20<br>21<br>22 | 2:30– 2:45<br>2:45– 3:00<br>3:00– 3:15<br>3:15– 3:30 | 2.9*<br>1.0<br>3.6*<br>3.0* | 29.4 | 4.7 | 0.5 | 1.4 | 10.0 | 11.9 | 83.4 |

* Dog restless.

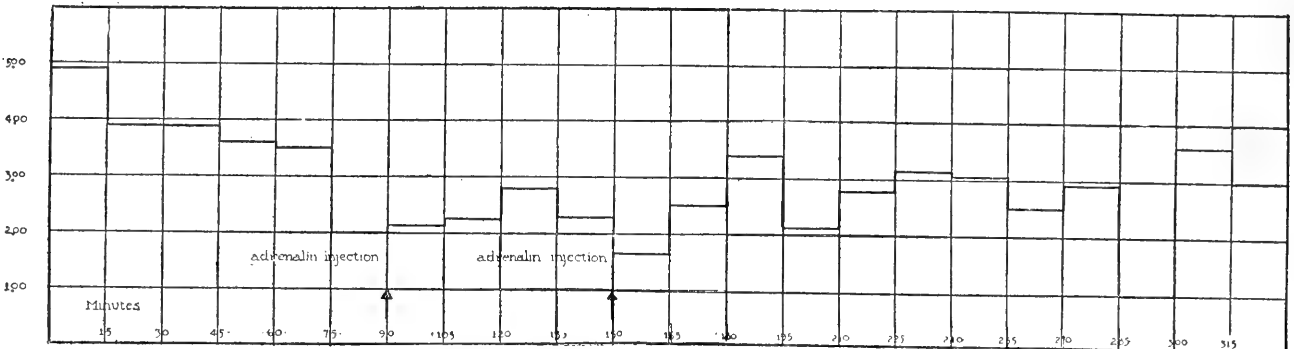


Chart 5. Dog 419. Effect of Small Doses of Adrenalin on the Lymph Flow

*Conclusion.* With small doses of epinephrin it seems probable that the primary stimulation of endothelium is negligible (no primary increase in permeability) while the effect in the later lessening of permeability is still evident in the diminution of the total protein as well as the relative increase in albumin.

In the following two experiments the same general effects of epinephrin on the lymph proteins is illustrated and in addition the effect on the phosphates, sugar and $CO_2$ tension of the lymph, as well as the electrical resistance.

*No. 444. Weight 8800*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | SUGAR | PHOSPHATES | BILE PIGMENT |
|---|---|---|---|---|---|---|---|
| | *minutes* | | *mgm.* | *per cent* | | | |
| 1 | 15 | 1.7 | 38.9 (1–2) | 5.1 | 118 | 6.1 | Trace |
| 2 | 15 | 1.9 | | | 118 | | |
| Epinephrin 5 cc. 1:10,000 solution intravenously | | | | | | | |
| 3 | 5 | 1.85 | | | | | |
| 4 | 5 | 1.5 | 38.3 (3–5) | 6.0 | 129 | 5.7 | |
| 5 | 5 | 1.35 | | | | | |
| 6 | 15 | 2.8 | 37.0 | 5.6 | 186 | 5.7 | Trace |
| 7 | 15 | 1.7 | 30.5 | 7.8 | 169 | 5.7 | |

*No. 471 January 22, 1923. Weight 16000*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | FIBRIN PER CUBIC CENTIMETER | SUGAR | $CO_2$ | RESISTANCE | PHOSPHATES |
|---|---|---|---|---|---|---|---|
| | *minutes* | | *mgm.* | | | | |
| 1 | 15 | 3.3 | 1.6 | 166 | 55 | 4.76 | 4.42 |
| Epinephrin 5 cc. 1 : 5000 intravenously | | | | | | | |
| 2 | 5 | 6.0 | 1.6 | 168 | 50 | 4.8 | 4.28 |
| 3 | 5 | 5.3 | 1.5 | 184 | 46 | 4.54 | 3.80 |
| 4 | 5 | 2.4 | | 208 | 45 | | 4.13 |
| 5 | 10 | 2.75 | 1.67 | 250 | 42 | 4.73 | 3.59 |
| 6 | 10 | 2.8 | 1.66 | 273 | 40 | 4.76 | 3.59 |
| 7 | 10 | 2.85 | 1.03 | 188 | 36 | 4.72 | 3.59 |
| 8 | 15 | 1.9 | 1.5 | 190 | 40 | 4.72 | 3.61 |
| 9 | 30 | 2.5 | 1.43 | 166 | 40 | 4.75 | 3.61 |

*Comment.* It will be observed that in both experiments the phosphates are reduced. The maximum ionization appears to take place very shortly after the injection of the epinephrin, resistance dropping from 4.8 to 4.54 and indicating, we believe, that the increased permeability indicated by this reduction takes place early and transiently to be followed by a marked reduction in the permeability of the endothelium. It will be noted that the greatest degree of acidity coincides in a general way with the maximum output of sugar.

### HEMOGLOBIN EXPERIMENTS

Experiments made with hemoglobin injections were carried out in a number of animals. In most of these the time was recorded when the hemoglobin was first apparent in the lymph after intravenous injection. These are shown in the following table:

Controls:

Dog 420: 5 minutes
Dog 421: 20 minutes
Dog 423: 20 minutes
Dog 424: 10 minutes
Dog 435: 10 minutes
Dog 436: 10 minutes
Average 12.5 minutes

Epinephrin animals:

Dog 436a: 25 minutes. This animal received 12 cc. of a 1:25,000 solution of epinephrin 1 hour before hemoglobin.
Dog 433: 35 minutes. This animal received 2 cc. of a 1:20,000 solution of epinephrin 1 hour before hemoglobin.
Dog 432: 20 minutes. This animal received 1 cc. of a 1:10,000 solution of epinephrin 1 hour before hemoglobin.
27 minutes, average.
Dog 430: 25 minutes. This animal received 1 cc. of a 1:10,000, 18 hours before injection.
Dog S3: 20 minutes. This animal received 2 cc. of a 1:10,000, 18 hours before injection.
Dog 428: 15 minutes. This animal received the hemoglobin and epinephrin at the same time. Epinephrin 1 cc. of 1:10,000.

*Conclusion.* In these animals the effect of the epinephrin injections was apparent in delaying the appearance of the hemoglobin in the lymph. This effect is, we believe, quite prolonged, even after 18 hours there seems to be occasional evidence of retardation. When added directly to the hemoglobin the dye comes through within normal limits.

In a second series we have colorimetrically measured the amount of hemoglobin that passes into the lymph stream. The titrations were made as follows: After the injection of the hemoglobin solution, blood was drawn and clotting prevented with oxalate crystals. Blood samples collected at later intervals as well as the samples of lymph, were compared colorimetrically to the original amount of hemoglobin present immediately after the hemoglobin injection. We present one chart (chart 6) in which has been plotted the curve for a normal animal and for the findings in the lymph after epinephrin injection (12 cc. intravenously, of a 1:25,000 solution) one hour before the injection of hemoglobin.

It will be observed from the chart that the amount that appears in the lymph of the epinephrin dog is about one-half the concentration that is found in the normal and the color is much delayed in its first appearance.

*Conclusion.* From a number of experiments carried out in which the amount of hemoglobin present in the lymph has been titrated colorimetrically, it has been found that the amount in the lymph is much less than when injected into the normal animal, indicating a lessened permeability to hemoglobin, and presumably to other molecular aggregates of the same or larger size.

## DISCUSSION

In the foregoing experiments we believe that we have demonstrated that the effect of epinephrin on the capillary endothelium is twofold, as far as the effect on the ultrafiltration through the cells is concerned. The effect depends to some extent on the dose but in general the primary effect is one of stimulation, with a transient increase in the permeability of the cells: this

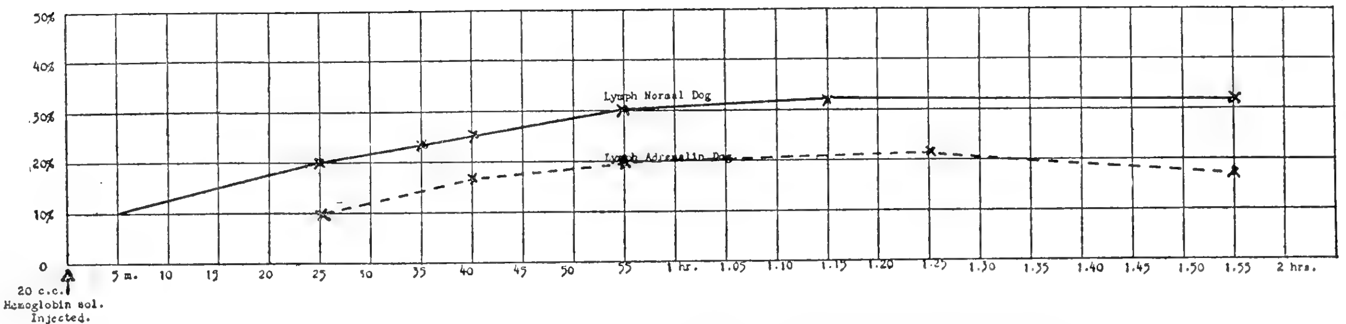


CHART. 6 HEMOGLOBIN CONCENTRATION IN LYMPH AFTER INTRAVENOUS INJECTION

Curve represents the percent of hemoglobin (the original concentration immediately after injection into the blood stream regarded as 100%).

is promptly followed by a period during which the endothelium is less permeable. This latter period is relatively prolonged and it is this phenomenon that is at the basis of a number of the therapeutic effects which are observed after epinephrin injection. Langer in his work has observed that the effect of the epinephrin in lessening permeability can be observed in the muscle cells when using a dilution of 1:10,000,000 and he found that the effect persisted for a considerable time after the epinephrin has been removed. This would correspond to the finding that we have here reported.

We might suppose that the effect of adrenalin on the capillary permeability would involve stimulation of the sympathetics, rather than a direct effect on the endothelial cell. We unfortunately know very little at the present time concerning the innervation of capillaries, particularly of the splanchnic area, although the general effect of splanchnic stimulation probably results in a contraction of the capillary wall (Krogh, 45). Such a contraction would render the wall less permeable.

Asher's (47) recent work seems to offer direct evidence that the nervous control of the capillary wall may have a direct effect on the permeability and thus brings physiological proof of the clinical contention of the importance of trophic nerves.

Even if we assume that epinephrin may alter the permeability of the capillary wall directly, as it seems to alter the muscle cell, we would by no means underestimate the importance of the findings of Molitor and Pick (47) who have ascertained that the hepatic circulation in carnivora is greatly impeded after epinephrin injections. We believe however, that such effects would not account for the long continued effect that we have occasionally observed. Lamson (48) accounts for the loss of fluid from the blood on an increase in filtration pressure in the liver, a view similar to that of Molitor and Pick.

Therapeutically epinephrin has found its field of greatest usefulness in the group of diseases that we associate with sensitization-asthma, hay fever, urticaria, angioneurotic edema, etc., and secondly in vascular disturbances associated with a loss of vascular tone.

When we deal with toxic substances circulating in the blood stream they may produce symptoms of intoxication because of direct injury to the endothelium, or having passed through the endothelium, are enabled to reach certain vital cell complexes in sufficient concentration to produce symptoms (as in the central nervous system, the bronchial musculature of the sensitized guinea-pig, etc.). Where, presumably, we are dealing with larger molecular aggregates we might assume that if we can make the endothelium less permeable, or make the specific cell complexes involved in the intoxication, less permeable, then our evidences of intoxication should be diminished. With lessened permeability, lessened susceptibility. Among the agents that we possess that seem to have this peculiar effect are to be mentioned the calcium salts (Starkenstein, 49) anesthetics in certain concentration, and epinephrin. We are of the impression that the effect of epinephrin in shock, whether surgical as described by Cannon (50) or following burns (Plaza, 51) (Pfeiffer, 52) is to be sought less in the pressor effect than in the lessening in the permeability of the cell membranes. Increased blood pressure with a coincident increase in permeability of the capillaries would result only in forcing more fluid from the vascular bed. The primary improvement must lie in the change in the permeability of the capillaries.

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# STUDIES IN ENDOTHELIAL PERMEABILITY. II

## THE RÔLE OF THE ENDOTHELIUM IN CANINE ANAPHYLACTIC SHOCK

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The fact that anaphylactic shock in the dog is associated with phenomena largely neuroendothelial in character as contrasted to the neuromuscular reaction that predominates in the guinea pig has led to an extensive study of these alterations in the vascular relations. It has been found that the effect on the lymph flow is quite similar to that when lymphagogues of 1st class are injected, i.e., an increased flow of lymph that is more concentrated in solids. This has been ascribed to the increased intraportal pressure. This increase in intraportal pressure has been assumed by some to be due to factors in which the liver is not directly participating, by others, such as Mautner and Pick (1) and Simmonds (2), actual vasoconstriction in the liver is held to be causative. Jaffe (3) has demonstrated smooth muscle fibers about the smaller veins in the liver and states that they are seen most easily when the liver is engorged and the capillary spaces distended.

For our purpose it is sufficient to emphasize that the participation of the neurovascular apparatus in shock due to anaphylaxis seems self-evident (one needs but recall the associated phenomena urticaria, hay fever, angeoneurotic edema, etc., in the human) and that we might anticipate that the endothelium will be a point of attack for the injuring agent, a position that Manwaring (4) has recently taken. That the endothelium would be merely a passive participant in the phenomena that have

been observed to occur in the lymph flow seems to us unwarranted. While the intrahepatic pressure is increased, such an increase would not in itself be sufficient to produce the effects.

In the following protocols we present the finding on 4 dogs which illustrate a number of the more interesting alterations that we have noted. We have, as in the previous experiments, used medium sized dogs; have made a thoracic fistula under local anesthetic, and have analyzed the lymph by means of the turbidimetric method which we have described in the first paper. We have carried out but one experiment with hemoglobin which demonstrated the immediate permeability of the capillaries for hemoglobin after egg albumin was injected in the sensitive animal. The animals were all sensitized by three intravenous injections of a fresh solution of egg-white given for three successive days. Reinjection was made not before two weeks after the last sensitizing injection.

*Effect of small dose.* Dog 391. Two cubic centimeters of a 20 per cent solution of fresh egg white injected intravenously.

PROTOCOL 1

*Lymph alterations in dog following small reinjection dose*

Anaphylaxis egg albumin: Final sensitizing dose August 25, 1922; experiment September 8, 1922; dog 391, weight 7 kilos

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| TIME | VOLUME | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDO-GLOBULIN I | PSEUDO-GLOBULIN II | ALBUMIN |
| | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 3 hours | 16.1* | 34.27 | 3.3 | 1.4 | 37.2 | 0 | 58.1 |
| Injected 2 cc. egg albumin (20 per cent solution) at 1:05 p.m. | | | | | | | |
| 1:00–1:15 | 4.5 | 43.75 | 5.7 | 1.1 | 46.6 | 0 | 46.6 |
| 1:15–1:30 | 11.5 | 50.2 | 7.1 | 1.6 | 41.5 | 4.2 | 45.6 |
| 1:30–1:45 | 7.0 | 47.0 | 9.0 | 0.6 | 38.0 | 7.0 | 45.4 |
| 1:45–2:00 | 8.5 | 40.9 | 7.8 | 0.7 | 42.8 | 5.0 | 43.7 |
| 2:00–2:15 | 5.5 | 34.8 | 9.2 | 0.8 | 47.2 | 0 | 42.8 |

* Equals 1.35 per fifteen minutes.

*Notes.* It will be observed that a well marked lymphagogue effect followed the injection, the flow increasing from a normal of about 1.35 cc. per fifteen minutes to 11.5 cc. shortly after the injection. An immediate increase in the total nitrogen of the lymph takes place. There was some delay in the clotting time of the first samples taken after the injection, but the fibrin finally formed a very solid clot. The relative amount of globulin increased. At the end of one hour the amount of nitrogen contained in the lymph had reached the preinjection level, although the rate of flow was still increased and the increase in the fibrin and globulin level was maintained.

*Conclusion.* A moderate shock in this animal resulted in distinct evidence of an increase in endothelial permeability that takes place immediately after the injection. Within one hour this effect begins to wear off but the composition of the lymph proteins remains altered in that a large increase in fibrinogen is maintained as well as a relatively larger amount of globulin than before the injection. The increase in the larger molecular aggregates (fibrinogen and the globulins) would indicate a well marked liver stimulation without the simultaneous mobilization of large amounts of proteolytic enzyme. It is primarily stimulation and not injury which becomes evident with these small doses. The increase in fibrinogen in these sensitized animals is a relatively common observation whenever the shock produced is not great. Whether it is associated with the general alteration in liver metabolism that takes place with sensitization that has been observed by Hashimoto and Pick (5) is not determined.

*Effect of moderate doses.* When we now turn to the effect of larger doses of egg albumin with which the shock effect was more apparent we find modifications of the lymph which probably have their origin in the enzyme mobilization which is associated with shock, Jobling and Petersen (6), Pfeiffer (7) and in the cell injury that probably takes place in the liver. In the following protocol the effect of a dose of 20 cc. of egg albumin solution is shown.

PROTOCOL 2

*Lymph alterations in dog following moderate dose of albumin*

Anaphylaxis egg albumin: Final sensitizing dose August 1, 1922; experiment August 17, 1922; dog 382, weight 18 kilos

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NUMBER | TIME | VOLUME | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | TOTAL FIBRIN AND EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | ALBUMIN |
| | | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 1–2<br>3–4 | 10:00–10:15<br>10:15–10:30 | 10.5<br>10.1 | 51.1 | 3.9 | 0.5 | 4.4 | 28.0 | 0 | 67.6 |
| 5–6<br>7–8 | 10:30–10:45<br>10:45–11:00 | 11.0<br>10.2 | 51.0 | 3.7 | 0.7 | 4.4 | 25.0 | 0 | 70.6 |
| Injected 20 cc. egg albumin solution (20 per cent) | | | | | | | | | |
| 9 | 11:00–11:07 | 18.5 | 51.5 | 1.1 | 7.0 | 8.1 | 28.7 | 0 | 63.2 |
| 10 | 11:07–11:15 | 26.0 | 65.5 | 0 | 5.4 | 5.4 | 19.0 | 0 | 75.6 |
| 11 | 11:15–11:22 | 26.0 | 63.7 | 0 | 5.8 | 5.8 | 20.0 | 0 | 74.2 |
| 12 | 11:22–11:30 | 18.0 | 60.0 | 0 | 6.6 | 6.6 | 17.7 | 1.3 | 74.4 |
| 13 | 11:30–11:37 | 19.0 | 60.0 | 0 | | | Lost | | |
| 14 | 11:37–11:45 | 15.0 | 57.5 | 0 | 5.0 | 5.0 | 22.8 | 2.4 | 69.8 |
| 15 | 11:45–11:52 | 12.0 | 56.4 | 0 | 6.2 | 6.2 | 19.0 | 0 | 74.8 |
| 16 | 11:52–12:00 | 13.0 | 57.5 | 0 | 6.0 | 6.0 | 17.0 | 0 | 77.0 |
| 17<br>18 | 12:00–12:15 | 24.0 | 56.6 | 0.6 | 4.4 | 5.0 | 20.0 | 0 | 74.4 |
| 19<br>20 | 12:15–12:30 | 25.0 | 57.5 | 1.5 | 4.1 | 5.6 | 20.0 | 0 | 74.4 |
| 21<br>22 | 12:30–12:45 | 25.0 | 56.2 | 6.0 | 3.5 | 9.5 | 26.0 | 0 | 64.5 |
| 23<br>24 | 12:45– 1:00<br>1:00– 1:15 | 16.0<br>20.0 | 56.3 | 6.2 | 3.2 | 9.4 | 22.0 | 0 | 68.6 |
| 25<br>26 | 1:15– 1:30<br>1:30– 1:45 | 18.0<br>14.5 | 52.4 | 5.0 | 2.8 | 7.8 | 24.0 | 0 | 68.2 |
| 27<br>28 | 1:45– 2:00<br>2:00– 2:15 | 14.0<br>11:2 | 51.7 | 5.0 | 3.2 | 8.2 | 26.0 | 0 | 65.8 |
| 29<br>30 | 2:15– 2:30<br>2:30– 2:45 | 17.5<br>12.0 | 49.0 | 4.7 | 2.2 | 6.9 | 23.0 | 0 | 70.1 |

Chart 1 illustrates the rate of lymph flow after the injection of 20 cc. of fresh egg albumin solution.

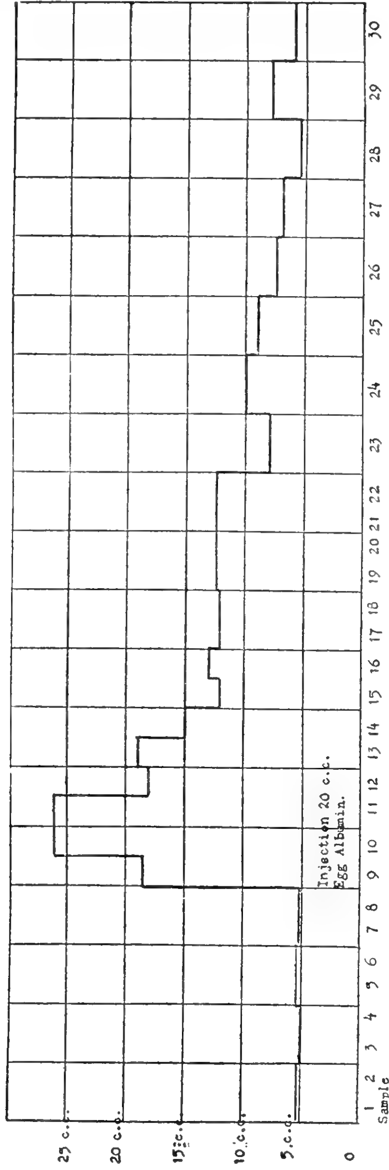


CHART 1. DOG 382. EFFECT OF ANAPHYLACTIC SHOCK ON RATE OF LYMPH FLOW

*Notes.* It will be observed that the lymph flow increased about fourfold and that the amount of protein increased from preinjection level of 51 to 65 mgm. per cubic centimeter. Clotting was inhibited, but during this time the larger molecular aggregates that precipitate with 14 per cent sodium sulphate increased greatly. Column 6 brings both the totals for fibrin and euglobulin. Pseudoglobulin I increased immediately after the injection, then diminished.

Pseudoglobulin II, previously absent, appeared during the course of the experiment, as in the preceding experiment. Three and one-half hours after the injection the protein concentration had diminished to a level somewhat less than the preinjection level.

*Conclusion.* It will be observed that the immediate reaction involved the same increase in flow with a relative increase in the larger molecular aggregates (euglobulin, pseudoglobulin I and II) and a relative decrease in the albumin. This would correspond to the usual effect of an increased permeability. Here, however, a change takes place from the usual picture because globulins now diminish and the albumins increase, the alteration persisting for about two hours. It is during this time interval that a sharp increase in proteolytic enzymes can be determined in the plasma and lymph and occasionally a considerable increase in hydrogen ion concentration, making conditions ideal for proteolysis. Stephan and his associates (8) have recently called attention to the association of the period during which blood is non-coagulable in shock to the mobilization of proteolytic enzymes. According to his conception the enzymes take an active part in preventing coagulation by hydrolyzing the larger (fibrinogen) aggregates to lower split products and in this manner preventing the formation of a clot. The experiment would seem to bear out this conception. Bulger (9) had previously associated the increased mobilization of proteolytic enzymes during shock with the incoagulability of the blood, finding that the antithrombin was not increased sufficiently to account for the picture.

PROTOCOL 3

*Lymph alterations in dog following moderate reinjection dose.*

Anaphylaxis—egg albumin: Final sensitizing dose August 25, 1922; experiment September 6, 1922; dog 390, weight 9.5 kgm.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| TIME | VOLUME | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | TOTAL FIBRIN AND EUGLOBULIN | PSEUDO-GLOBULIN I | PSUEDO-GLOBULIN II | ALBUMIN |
| | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 11:15–11:30<br>11:30–11:45 | 5.2<br>4.2 | 36.16 | 3.0 | 0 | 3.0 | 38.7 | 0 | 58.3 |
| 11:45–12:00<br>12:00–12:15 | 5.2<br>6.1 | 36.16 | 3.0 | 0.4 | 3.4 | 39.5 | 0 | 57.3 |
| Injected 20 cc. egg albumin (20 per cent) at 12.15 | | | | | | | | |
| 12:15–12:22 | 20.4 | 48.77 | 0 | 3.2 | 3.2 | 33.7 | 0 | 63.1 |
| 12:22–12:30 | 18.5 | 51.12 | 1.7 | 2.4 | 4.1 | 28.3 | 2.7 | 64.9 |
| 12:30–12:45 | 31.7 | 54.65 | 2.7 | 1.7 | 4.4 | 20.7 | 3.6 | 71.3 |
| 12:45– 1:00 | 17.0 | 49.36 | 5.5 | 0.5 | 6.0 | 27.9 | 0 | 66.1 |
| 1:00– 1:15<br>1:15– 1:30 | 13.0<br>11.0 | 46.05 | 5.1 | 0.7 | 5.8 | 31.1 | 0 | 62.1 |
| 1:30– 1:45<br>1:45– 2:00 | 18.3<br>16.4 | 45.45 | 5.5 | 0.9 | 6.4 | 34.6 | 0 | 59.0 |
| 2:00– 2:15<br>2:15– 2:30 | 10.2<br>7.3 | 43.84 | 5.3 | 0.7 | 6.0 | 30.0 | 3.2 | 60.8 |
| 2:30– 2:45<br>2:45– 3:00 | 6.0<br>5.0 | 41.99 | 4.6 | 0.2 | 4.8 | 34.3 | 1.8 | 59.1 |
| 3:00– 3:15<br>3:15– 3:30 | 6.6<br>8.2 | 39.07 | 3.3 | 0 | 3.3 | 33.3 | 8.7 | 54.7 |
| 3:30– 3:45<br>3:45– 4:00 | 7.5<br>6.2 | 30.32 | 3.9 | 0 | 3.9 | 35.8 | 2.0 | 58.3 |

*Notes.* It will be observed that in this animal the total amount of protein nitrogen, after increasing in the usual manner, had diminished at the end of a four hour period of observation, to somewhat less than the preinjection level, the volume at this

time being almost at the normal rate. Clotting, while delayed, took place in practically all the tubes except the first one after the injection. Euglobulin, at first greatly increased, diminished rapidly. The smaller globulin fractions were never increased in amount except pseudoglobulin II which increased as in the two preceding experiments.

*Conclusion.* The general effects are similar to those observed in the previous experiment except that here the primary alteration in the globulin-albumin ratio seen in the preceding experiments is no longer found. The same increase in the relative amount of albumin is seen here as in the preceding animals, although the maximum is reached here in thirty minutes instead of in one hour as in dog 382. Preinjection levels are reached in about three hours.

We wish finally to present the picture obtained when a large fatal dose of egg albumin is injected in a sensitized animal. The animal died two hours after the injection.

PROTOCOL 4

*Lymph and blood plasma alterations following fatal reinjection dose*

Anaphylaxis—egg albumin: Final sensitizing dose August 1, 1922; experiment August 18, 1922; dog 400, weight 9, kilos

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| TIME | VOLUME | TOTAL PROTEIN | FIBRIN | EUGLOBULIN | TOTAL FIBRIN AND EUGLOBULINS | PSEUDOGLOBULIN | PSEUDOGLOBULIN II | ALBUMIN |
| | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 12:00–12:30 | 12.8 | 46.0 | 3.0 | 0 | 3.0 | 17.1 | 1.7 | 78.2 |
| 12:30– 1:00 | 13.8 | 46.18 | 3.4 | 0.6 | 4.0 | 17.6 | 0.8 | 77.6 |
| Injected 80 cc. 20 per cent egg albumin solution intravenously | | | | | | | | |
| 1:00– 1:05 | 5.2 | 54.8 | 0.3 | 2.4 | 2.7 | 15.2 | 0 | 82.1 |
| 1:05– 1:15 | 26.5 | 54.6 | 0 | 2.5 | 2.5 | 15.0 | 1.1 | 81.4 |
| 1:15– 1:30 | 51.0 | 58.25 | 0 | 2.3 | 2.3 | 13.3 | 3.4 | 81.0 |
| 1:30– 2:00 | 49.5 | 56.21 | 0 | 2.4 | 2.4 | 14.2 | 1.5 | 81.9 |
| 2:00– 2:30 | 27.3 | 54.9 | 0.5 | 2.4 | 2.6 | 12.3 | 3.4 | 81.7 |
| 2:30– 3:00 | 11.8 | 52.8 | 1.5 | 1.5 | 3.0 | 13.0 | 4.2 | 79.8 |
| Died | | | | | | | | |
| Plasma (blood) | | | | | | | | |
| Before injection | | 48.41 | 2.0 | 3.4 | 5.4 | 15.3 | 3.1 | 76.2 |
| 1.30 | | 43.68 | 0 | 3.2 | 3.2 | 14.3 | 3.3 | 79.2 |
| After death | | 45.37 | 0 | 1.6 | 1.6 | 22.21 | 3.0 | 73.0 |

*Notes.* The results are similar to the preceding with the following exceptions: The total larger molecular aggregates (fibrin, euglobulin, and pseudoglobulin I) are all diminished immediately after the injection but pseudoglobulin II is increased even more than in the former three protocols. Analysis made of the blood plasma during the course of the experiment reveals that the actual amount of protein in the lymph was almost as great at the beginning of the experiment and that after the shock the amount of protein in the lymph was actually greater than the amount in the blood plasma. Part of the relative dilution of the blood plasma was no doubt due to the large amount of fluid injected.

*Conclusion.* We believe that in this experiment the enzymatic changes (in the lymph, and intracellular) must largely account for the fact that the larger molecular aggregates are lessened in amount while the albumin and smaller fragments are increased. We believe that the increase in pseudoglobulin II, which is apparent in each of these experiments, can be interpreted in this way. The fact that in this experiment the actual amount of protein in the lymph was finally greater than that in the blood plasma would seem to give further evidence of an extensive destruction of protein. It is of course not excluded that the increased hydration of the tissues through which the lymph flows may attract water and result in making the lymph relatively rich in protein. Perhaps both factors, so closely related in many ways, are operative.

## DISCUSSION

Inasmuch as we have found no comparable alterations in the lymph in animals that were injected with protein when not sensitive we believe that the alterations revealed afford some insight into changes that take place in shock due to anaphylaxis. In all of the animals the onset of the immediate lymphagogue effect was associated with the prompt passage of erythrocytes into the lymph stream and, when the shock was severe, with hemolysis. In the animals shocked with a moderate dose, bile pigments made their appearance some time after the injection.

The sudden stimulation of the endothelium and the associated increase in permeability is quite apparent from a mere analysis of the lymph. We have therefore made but few experiments with hemoglobin injections (which because of the associated hemolysis due to the destruction of the red blood corpuscles of the dog has but a limited value here) and these showed that the hemoglobin came through within five minutes after injection; that is, within the time period at which crystalloids would appear, indicating the marked increase in permeability which had taken place.

It seems evident that in shock of this type we deal with a stimulation of the endothelium so great that we may speak of fatigue of the cells; this may go over to death. Recovery may take place, in our animals in from one to four hours, depending on the dose. If recovery does take place we might anticipate that the endothelium will later be relatively less susceptible to a further change. This is probably one of the phenomena involved in the refractory state apparent after anaphylactic and other forms of shock.

Indeed, that the severity of the reaction plays some rôle in this resistance seems indicated in the experiments of Karsner and Ecker (10). They found that animals that were shocked with heterologous and homologous antigens were, as a rule, resistant to a following shock to a degree commensurate with the amount of reaction experienced during the primary shock. Thus the animals injected with a homologous antigen the first time were, as a rule, more resistant to a following shock than animals treated first with a heterologous antigen, which, as a rule, did not induce much reaction the first time.

We are of the impression that the great mobilization of proteolytic and other enzymes that takes place in shock and which probably have their origin in (and diffuse out from) cells that have been stimulated, or injured (such as the endothelium, liver, pancreas, etc.) must have a decided effect in breaking up the larger molecular aggregates (fibrinogen, globulins) almost as rapidly as they are thrown into the circulating fluids and perhaps even before (intracellular).

As far as the effect on the fibrinogen is concerned we observed the most marked mobilization with the smallest dose of protein (to 9 per cent); the least amount with the fatal dose, with a combined maximum of 3 per cent for fibrinogen and euglobulin. The effect here must depend on liver stimulation in the case of the small dose with a resulting maximum reaction; in the fatal case there appears to be an actual destruction of these larger fragments before they are thrown into the circulation.

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# STUDIES IN ENDOTHELIAL PERMEABILITY. III

## THE MODIFICATION OF THE THORACIC LYMPH FOLLOWING PORTAL BLOCKADE

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In the preceding paper we have pointed out that in anaphylactic shock in the dog the lymph proteins show considerable modification, depending to a large extent on the dose used for reinjection. From previous studies that we have made with the enzyme mobilization during anaphylactic shock and from the work of Dale (1), Manwaring (2) and others, it seems most probable to us that the lymph proteins are modified by several factors. First, the alteration in the permeability of the endothelium which permits a large amount of blood plasma suddenly to flood into the lymph spaces of the mesenteric area, especially with the increased intraportal pressure which is at once evident in anaphylactic shock. Second, the proteins derived from the liver and other abdominal viscera under shock conditions; these would include particularly the larger molecular aggregates, fibrinogen, globulins, etc. Third, the digestion of these proteins which may begin intracellularly in an explosive manner and be continued during their passage from the liver cells to the lymph stream. This seems most evident, as we might anticipate, in the more severe cases of shock in which we find that the lymph proteins are present rather in the smaller than in the larger molecular aggregates.

In the work of Pick, Simonds, Manwaring and others, the importance of the alterations of the intraportal pressure has been emphasized and the great engorgement of the liver brought into causal relationship with certain phases of anaphylactic shock. That the effect of a purely physical alteration in the liver circulation might be studied in connection with the anaphy-

lactic pictures that we have obtained from the thoracic lymph, we have carried out a number of experiments of the following nature:

A lymph fistula was established under local anesthetic as usual, then a small abdominal incision was made under local anesthetic and 10 cc. of liquid petrolatum coarsely emulsified in dog serum, was injected intraportally. Inasmuch as the results in these experiments have been quite uniform, we show only one protocol.

PROTOCOL 1

*No. 476; February 5, 1923 weight 10.5 kilos; Intraportal oil, injection*

| NUMBER | TIME | VOLUME | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS | TOTAL FIBRIN AND GLOBULIN | ALBUMIN | SUGAR | $CO_2$ | BILE PIGMENT | PHOSPHATE | PROTEASE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | *per cent* | | | | | | | |
| 1 | 10:30–10:45 | 7.5 | 42.2 | 6.1 | 1.5 | 16.7 | 24.3 | 75.7 | 147 | 0.54 | 0 | 4.72 | 0 |
| 2 | 10:45–11:00 | 8.3 | 42.5 | 6.8 | 1.2 | 16.7 | 24.7 | 75.3 | 157 | 0.41 | 0 | 4.92 | 0 |
| 10 cc. liquid petrolatum intraportal injection at 10.55 | | | | | | | | | | | | | |
| 3 | 11:00–11:07* | 6.0 | 36.0 | 5.0 | 1.5 | 18.1 | 24.6 | 75.4 | 161 | 0.55 | 0 | 4.63 | 0 |
| 4 | 11:07–11:15 | 9.6 | 27.4 | 4.7 | 2.1 | 17.1 | 23.9 | 76.1 | 188 | 0.55 | 0 | 4.43 | 0 |
| 5 | 11:15–11:30 | 26.6 | 26.8 | 4.8 | 2.0 | 18.6 | 25.4 | 74.6 | 190 | 0.56 | 0 | 4.27 | 0 |
| 6 | 11:30–11:45 | 23.3 | 35.0 | 4.8 | 2.4 | 17.3 | 24.5 | 75.5 | 200 | 0.60 | 0 | 4.13 | 0 |
| 7 | 11:45–12:00 | 20.0 | 35.3 | 5.6 | 2.4 | 19.5 | 27.5 | 72.5 | 258 | 0.42 | + | 5.0 | 0 |
| 8 | 12:00–12:15** | 17.7 | 35.0 | 5.0 | 2.4 | 21.7 | 29.1 | 70.9 | 259 | 0.62 | + | 5.95 | 0 |
| 9 | 12:15–12:30 | 17.0 | 36.4 | 5.2 | 3.4 | 18.0 | 26.6 | 73.4 | 277 | 0.26 | + | 6.83 | + |
| 10 | 12:30–12:45 | 14.7 | 34.9 | 4.9 | 2.7 | 20.7 | 28.3 | 71.7 | 312 | 0.36 | + | 6.17 | +++ |
| 11 | 12:45– 1:00 | 9.3 | 32.7 | 5.5 | 2.8 | 22.6 | 30.9 | 69.1 | 400 | 0.38 | + | 6.43 | +++ |
| 12 | 1:00– 1:30 | 5.1 | 31.3 | 7.0 | 2.5 | 22.3 | 31.8 | 68.2 | 571 | 0.30 | + | 5.96 | +++ |
| Plasma | | | | | | | | | | | | | |
| | 11:30........ | | 45.9 | 9.0 | | 23.3 | 32.3 | 67.7 | 166 | 0.40 | + | 4.24 | |
| | 12 noon....... | | | | | | | | 169 | 0.30 | | | |
| | 1:00.......... | | | | | | | | 350 | 0.29 | +++ | 6.35 | |

The dog died at 1:30 p.m. On opening the animal the liver was found rather small, hard, mottled. The splanchnic vessels were engorged. The spleen was very large and soft, weighing 85 grams. The lungs and heart were normal, with but little blood in the chambers of the heart.

* Red blood corpuscles appear.

** Red blood corpuscles appear in greater number.

The injection of an indifferent oil such as petrolatum should have little effect other than a mechanical one of blocking the liver capillaries, resulting in an increase in the intraportal pressure without primary injury of the splanchnic endothelium or the hepatic cells. The alterations shown in the protocol are of particular interest in connection with the observations recorded in the experiments with anaphylactic shock. There results first a marked lymphagogue effect reaching a maximum in about fifteen minutes after the injection. Here, then, with merely an increased intraportal pressure lymph filtration is obviously increased, but it differs from the effect in anaphylactic shock in that the protein of the lymph is not increased, but on the contrary, is diminished, indicating an unaltered condition of the endothelium, which in this case permits only a relatively thin lymph to pass through the capillaries. It is of interest to note furthermore that the relation between globulins and albumins is practically unaltered. Assuming that the fibrinogen comes from the liver, the reduction in the amount coming through after the injection can be explained on the assumption that much of our fluid is now coming through from the other parts of the splanchnic area, rather than from the liver. Sugar, $CO_2$ tension and phosphates remain practically unaltered.

Beginning with sample 5 we note a striking difference.

The protein in the lymph increases. In the following samples an increase in fibrinogen, in the globulins, in the sugar, the phosphates and in bile pigment is apparent, an acidosis occurs and proteolytic enzymes make their appearance in large amounts. Red blood corpuscles are also observed in considerable concentration. We now have all the evidences of a marked liver stimulation and injury, resembling in many respects that which we have observed both as a result of the primary injury after peptone injection and anaphylactic shock, or the secondary alteration after peptone injection to which we have called attention.

## DISCUSSION

These experiments with a portal blockade illustrate that with an increase in intraportal pressure we may have a lymphagogue

effect but the lymph coming into the thoracic duct under these conditions is one that is relatively poor in protein. There are no other alterations of moment. When, however, injury to tissue results because of the disturbed vascular conditions, particularly in the liver, and capillary injury has presumably taken place as a result of stasis, marked alterations take place. The picture differs from severe shock only in the fact that the coagulation mechanism of the lymph remains unaltered and that evidently proteolytic enzymes are not thrown into the circulation as rapidly and to the same extent that we find them in peptone and anaphylactic shock. The differences can probably be accounted for on the basis that not all parts of the liver parenchyma are equally involved in the change, some portions may be stimulated, in others the stimulation may have gone over to fatigue and actual distribution of cells.

In view of the fact that in the various shock effects in the dog a passive hyperemia of the liver is usually marked, we think it possible that some of the alterations that we have observed under such conditions are not induced primarily by the injuring agent, but follow in the wake of the primary change and can be accounted for as a result of the vascular alterations. During the course of these secondary alterations it is quite possible that toxic substances may arise in the liver parenchyma undergoing degeneration, which, circulating in the body, may cause further injury and even death. In these experiments for instance, we are of the impression that the death of the animal is not wholly due to the disorganization of the vascular bed, but that an intoxication arising as a result of the liver injury has a large share in bringing about the rapid death of the animal.

While we have made no determinations of the intraportal blood pressure, it seems evident from a study of the protocol that the great alterations of the lymph (increased concentration of the protein, globulins, sugar, bile pigment, phosphate, etc.) which take place after sample 5 cannot be due to an alteration of intraportal pressure. Should the intraportal pressure fall the lymph might become more concentrated when larger amounts of water would reënter the capillaries. But it will be noted that

the volume of the lymph flow remains fairly constant. In tubes 6 to 10, for instance the volume diminished some 24 per cent but the phosphates increased 65 per cent. Such an alteration cannot be due to concentration, but must depend on the altered rate of diffusion from cells.

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# STUDIES ON ENDOTHELIAL PERMEABILITY. IV

## THE MODIFICATION OF CANINE ANAPHYLACTIC SHOCK BY MEANS OF ENDOTHELIAL BLOCKADE

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In the preceding paper evidence has been presented which points to the fact that in canine anaphylaxis the endothelium, particularly that of the liver, plays a very prominent rôle in the mechanism of shock. Indeed it might seem that the chief point of attack of the reinjected protein is in this tissue, and that after these cells have been injured and become more permeable the liver is the next organ to be affected. The whole phenomenon is a vascular shock rather than a neuro-muscular one such as we see in the guinea pig. This by no means excludes the possibility that toxic products may arise secondarily from the liver, as Manwaring (1) has contended; or that the mobilization of proteolytic enzymes, which has been previously demonstrated (2) may not act in producing toxic products from the disintegrating liver or other tissue cells.

If the endothelium is vitally concerned we might be able to cause some protection against shock in dogs if we produce an "endothelial blockade" by means of a previously injected colloid. Admittedly a crude procedure and one that at best can only partially alter the permeability of the cells, we have felt that it might at least offer us some confirmation of the rôle of the endothelium in shock.

The following sets of protocols of two series of animals will illustrate the results. In each series one of the control animals

died of distemper so that we can show but one normal control for each series, but the results are, we believe, sufficiently clear cut to warrant conclusions. We have carried out several determinations on the lymph not included in the first experiments which may be of interest, such as those of sugar, bile pigments, carbon dioxide, and inorganic phosphates.

## EXPERIMENTAL

### *First series*

All animals were given intravenous injections of 20 cc. of a 20 per cent solution of fresh egg albumin on successive days. They were then given a rest period of three days. They were then given daily intravenous injections of 15 cc. of a twenty per cent solution of saccharated red oxide of iron until they were used for the final shocking dose.

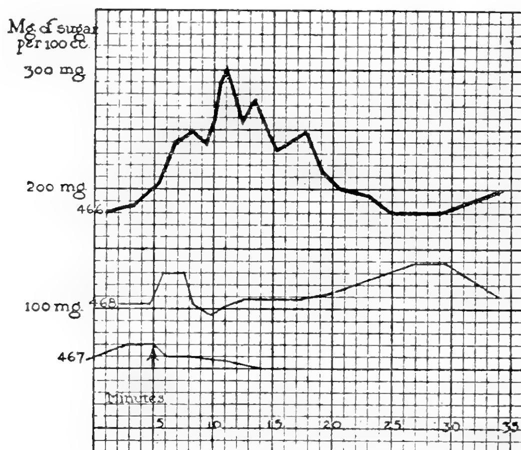


CHART 1. SUGAR CONTENT, FIRST SERIES

Heavy line, control animal. Light lines, animals protected by "endothelial blockade." Arrow indicates time of injection of shock dose.

The first protocol shows the effects of the shock dose of albumin in the control.

PROTOCOL 1

*No. 466; January 15, 1923; weight 8500; sensitization completed January 1, 1923; control animal; no iron oxide injections*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN AND EUGLOBULIN | PSEUDOGLOBULINS I AND II | FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | $CO_2$ | PHOSPHATES | BILE PIGMENT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *minutes* | | *mgm.* | | | | | | | | |
| 1 | 15 | 4.1 | 26.1 | 0.7 | 18.7 | 19.4 | 80.6 | 180 | 51.3 | 4.80 | 0 |
| 2 | 15 | 1.6 | 27.24 | 1.1 | 17.2 | 18.3 | 81.7 | 187 | 60.7 | 3.65 | 0 |
| 20 cc. 40 per cent fresh egg albumin solution intravenously | | | | | | | | | | | |
| 3 | 5 | 4.0 | 30.0 | 1.6 | 16.3 | 17.9 | 82.1 | 206 | 68.3 | 3.25 | 0 |
| 4 | 5 | 8.5 | 40.0 | 2.6 | 16.6 | 19.2 | 80.8 | 243 | 30.5 | 4.15 | 0 |
| 5 | 5 | 6.9 | 42.8 | 2.3 | 18.2 | 20.5 | 79.5 | 243 | 46.6 | 3.76 | 0 |
| 6 | 5 | 9.8 | 44.4 | 2.8 | 17.5 | 20.1 | 79.9 | 250 | 45.7 | 5.0 | 0 |
| 7 | 5 | 12.3 | 41.0 | 3.1 | 18.2 | 21.3 | 78.7 | 238 | 47.5 | 4.55 | 0 |
| 8 | 5 | 13.0 | 40.0 | 3.0 | 15.7 | 18.7 | 81.3 | 285 | 51.3 | 4.55 | 0 |
| 9 | 5 | 10.0 | 36.5 | 3.4 | 16.8 | 20.2 | 79.8 | 303 | | 4.13 | 0 |
| 10 | 5 | 8.8 | 33.6 | 3.1 | 19.0 | 22.1 | 77.9 | 250 | 49.4 | 4.35 | 0 |
| 11 | 5 | 8.0 | 34.2 | 3.0 | 15.5 | 18.5 | 81.5 | 285 | | 4.14 | 0 |
| 12 | 5 | 8.2 | 32.4 | 3.2 | 15.7 | 18.9 | 81.1 | | 51.3 | 5.85 | 0 |
| 13 | 5 | 7.4 | 30.7 | 2.7 | 13.3 | 16.0 | 84.0 | 227 | 47.3 | 4.86 | 0 |
| 14 | 5 | 5.6 | 30.7 | 3.0 | 14.8 | 17.8 | 82.2 | 240 | 39.0 | 3.95 | 0 |
| 15 | 5 | 6.5 | 30.0 | 3.7 | 13.3 | 17.0 | 83.0 | 250 | 46.6 | 3.70 | 0 |
| 16 | 5 | 5.1 | 29.2 | 3.7 | 11.8 | 15.5 | 84.5 | 250 | 41.9 | 3.70 | Trace |
| 17 | 5 | 6.7 | 27.5 | 3.4 | 13.8 | 17.2 | 82.8 | 215 | | 3.72 | + |
| 18 | 10 | 4.7 | 27.0 | 3.1 | 14.4 | 17.5 | 82.5 | 200 | 43.8 | 3.65 | + |
| 19 | 10 | 6.0 | 28.8 | 2.9 | 14.0 | 16.9 | 83.1 | 198 | | 4.25 | + |
| 20 | 10 | 3.8 | 27.4 | 3.0 | 15.4 | 18.4 | 81.6 | 181 | 43.8 | 3.95 | ++ |
| 21 | 30 | 2.3 | 27.0 | 2.2 | 13.1 | 15.3 | 84.7 | 181 | | 4.03 | ++ |
| 22 | 30 | 1.8 | 26.1 | 2.0 | 13.2 | 15.2 | 84.8 | 200 | 45.7 | 4.25 | +++ |

Red blood corpuscles appeared in the lymph immediately after the injection and were especially marked in samples 12 to 17. All samples were collected under petroleum oil and with the addition of sodium oxalate, as a consequence the time of coagulation of the lymph could not be noted.

The control animal shows that there was a well marked lymphagogue effect, increase in lymph protein, well defined increase in sugar, in phosphates and in bile pigment, and in occasional samples the tests for the carbon dioxide combining power showed evidence of acidosis. The alterations in the globulin-albumin ratio are those that have been described in the preceding paper as a result of moderately severe anaphylaxis.

The following protocols illustrate the effects in the two corresponding animals that received the colloidal iron injections.

PROTOCOL 2

| | MINUTES | VOLUME | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN AND EUGLOBULIN | PSEUDO-GLOBULINS | TOTAL FIBRIN AND GLOBULIN | ALBUMIN | SUGAR | PHOSPHATE | BILE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *cc.* | *mgm.* | | | | | | | |
| 1 | 30 | 1.2 | 31.8 | 2.4 | 19.7 | 22.1 | 77.9 | 62 | 4.2 | ++ |
| 2 | 30 | 1.9 | 29.6 | 2.4 | 21.6 | 24.0 | 76.0 | 71 | 4.1 | +++ |
| 20 cc. 40 per cent solution fresh egg albumin injected intravenously | | | | | | | | | | |
| 3 | 15 | 1.1 | 32.4 | 2.1 | 21.1 | 23.1 | 76.2 | 60 | 4.1 | ++++ |
| 4 | 15 | 1.4 | 33.0 | 2.5 | 21.3 | 23.8 | 76.9 | 58 | 4.3 | ++++ |
| 5 | 15 | 1.3 | 34.8 | 3.0 | 20.1 | 23.1 | 76.9 | 52 | 4.1 | +++ |

The animal responded with practically no change in lymph volume, only very minor changes in the concentration of protein, and no significant change in sugar, phosphates or carbon dioxide.

The protocol of the second anaphylaxic dog is on the following page.

Here, as with the previous animal of this series, which had been injected with colloidal ion, there resulted very little lymphagogue effect when the shock dose of albumin was injected, and there was little variation of the protein of the lymph. Sugar, as well as carbon dioxide, showed little change, and the phosphates remained quite constant except for a transient rise immediately after the injection, and during a later period (samples 7 and 8). Fluctuations in the albumin-globulin ratio were very marked. No red blood corpuscles appeared in the lymph.

PROTOCOL 3

*No. 468; January 16, 1923; weight 9500; sensitization completed January 1, 1923*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS I AND II | TOTAL FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | $CO_2$ | PHOSPHATE | BILE PIGMENT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *minutes* | | *mgm.* | | | | | | | | | |
| 1 | 15 | 2.93 | 62.6 | 4.1 | 11.8 | 50.5 | 66.4 | 33.6 | 105 | 57.9 | 3.44 | Faint trace |
| 20 cc. 40 per cent solution egg albumin intravenously | | | | | | | | | | | | |
| 2 | 5 | 3.8 | 63.0 | 3.0 | 14.7 | 27.0 | 44.7 | 55.3 | 128 | 56.0 | 4.23 | |
| 3 | 5 | 4.5 | 66.6 | 0 | 19.1 | 30.3 | 49.3 | 50.7 | 128 | 61.7 | 3.68 | Faint trace |
| 4 | 5 | 4.0 | 66.6 | 0 | 17.0 | 35.0 | 52.0 | 48.0 | 127 | 57.9 | 3.33 | |
| 5 | 5 | 3.7 | 63.0 | 0 | 15.4 | 32.3 | 47.7 | 52.3 | 101 | 57.9 | 3.63 | |
| 6 | 5 | 3.4 | 60.9 | 0.9 | 30.0 | 14.1 | 45.0 | 55.0 | 95 | 46.6 | 3.57 | |
| 7 | 5 | 3.5 | 55.2 | 0 | 17.8 | 28.0 | 45.8 | 54.2 | 100 | | 3.85 | + |
| 8 | 15 | 2.8 | 56.5 | 3.7 | 14.7 | 30.9 | 49.3 | 50.7 | 107 | 56.0 | 4.35 | Trace |
| 9 | 15 | 3.7* | 56.4 | 7.4 | 14.3 | 22.5 | 44.2 | 55.8 | 109 | | 3.13 | + |
| 10 | 15 | 3.8* | 58.5 | 6.8 | 13.1 | 38.4 | 58.3 | 41.7 | 112 | 56.0 | 3.17 | + |
| 11 | 15 | 3.8* | 51.4 | 2.7 | 13.6 | 34.2 | 50.5 | 49.5 | 119 | | 3.17 | + |
| 12 | 15 | 2.2 | 52.1 | 4.0 | 13.2 | 28.4 | 45.6 | 54.4 | 129 | 59.8 | 3.25 | + |
| 13 | 15 | 2.2 | 55.7 | 6.3 | 13.0 | 30.5 | 49.8 | 50.2 | 139 | | 3.09 | + |
| 14 | 15 | 1.7 | 58.0 | 17.2 | 13.8 | 28.1 | 59.1 | 40.9 | 136 | | 2.98 | ++ |
| 15 | 30 | 1.5 | 53.0 | 5.6 | 14.1 | 30.3 | 50.0 | 50.0 | 111 | | 2.91 | +++ |

* Animal restless.

## *Second series*

The second series is even more clear cut than the first. Animals were sensitized as in the first experiment, and were given daily intravenous injections of sacchrated iron up to the day they received the final shock dose.

The record of the control animal is on the following page.

The animal died forty-five minutes after the shock dose. Striking alterations are observed in the phosphates and the sugar, the carbon dioxide changes are relatively minor in character. Red blood corpuscles appear in great quantity, the final lymph having almost the appearance of pure blood. Apart from the changes in the coagulation, the proteins did not

## PROTOCOL 4

*No. 478; February 7, 1923; weight 9300; control*

| NUMBER | TIME | VOLUME | | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN | TOTAL FIBRIN AND GLOBULIN | ALBUMIN | SUGAR | $CO_2$ | BILE | PHOSPHATE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *mgm.* | *per cent* | | | | | | | | |
| 1 | 10:30–10:45 | 6.8 | | 32.5 | 2.1 | 0.4 | 14.1 | 16.6 | 83.4 | 124 | 48 | | 1.75 |
| 2 | 10:45–11:00 | 6.4 | RBC | 34.7 | 1.4 | 0.7 | 13.0 | 15.1 | 84.9 | 123 | 52 | | 1.89 |
| 3 | 11:00–11:15 | 7.3 | RBC | 32.5 | 2.1 | 0.4 | 13.8 | 16.3 | 83.6 | 161 | 56 | | 1.85 |
| 4 | 11:15–11:30 | 5.6 | RBC | | | | | | | | 58 | | 2.18 |
| 20 cc. 40 per cent solution of fresh egg albumin injected intravenously | | | | | | | | | | | | | |
| 5 | 11:30–11:35 | 8.2 | RBC* | 36.9 | 0 | 2.0 | 14.0 | 16.0 | 84.0 | 161 | 56 | Trace | 1.88 |
| 6 | 11:35–11:40 | 9.6 | RBC | 45.6 | 0 | 3.0 | 15.0 | 18.0 | 82.0 | 144 | 54 | Faint trace | 2.72 |
| 7 | 11:40–11:45 | 7.4 | RBC | 45.6 | 0 | 3.5 | 13.9 | 17.4 | 82.6 | 266 | 53 | Faint trace | 2.38 |
| 8 | 11:45–12:00 | 14.3 | RBC | 44.4 | 0 | 4.2 | 13.5 | 17.7 | 82.3 | 400 | 56 | Faint trace | 2.72 |
| 9 | 12:00–12:15 | 8.1 | RBC | 39.96 | 0 | 3.8 | 13.0 | 16.8 | 83.2 | 408 | 58 | Faint trace | 2.78 |
| Plasma | | | | | | | | | | | | | |
| 1 | 10:45 | | | | | 3.2 | 13.2 | 16.4 | 83.2 | 151 | 54 | | 1.67 |
| 2 | 11:20 | | | | | | | | | 227 | 50 | | 2.38 |
| 3 | 12:00 | | | | | | | | | | 56 | | 2.78 |

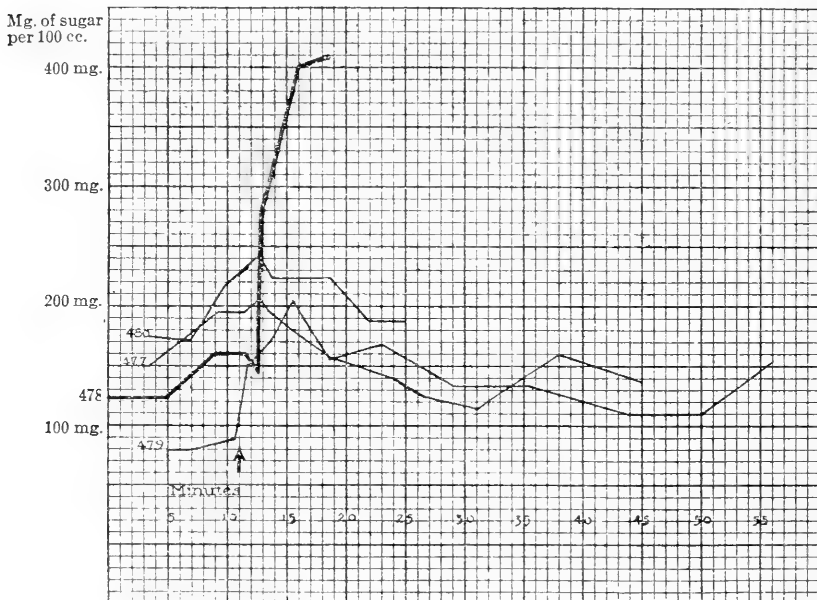


CHART 2. SUGAR CONTENT, SECOND SERIES

Heavy line, control animal. Light lines, animals protected by "endothelial blockade." Arrow indicates time of injection of shock dose.

greatly alter their relation. This may, perhaps, be due to a simultaneous liberation of large amounts of proteolytic enzymes which would break up the proteins having larger molecular aggregates.

The individual protocols follow:

PROTOCOL 5

*No. 477; February 6, 1923; weight 10200; dog sensitized, final dose January 22, 1923, and received colloidal iron, 9 doses*

| Number | Time | Volume per five minutes | Protein per cubic centimeter | Fibrin | Euglobulin | Pseudoglobulin | Total fibrin and globulin | Albumin | Sugar | $CO_2$ | Phosphates | Bile pigment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *minutes* | *cc.* | *mgm.* | *per cent* | *per cent* | *per cent* | | *per cent* | | | | |
| 1 | 15 | 1.0 | 45.0 | 12.0 | 1.5 | 15.3 | 28.8 | 71.2 | 150 | 60 | 3.05 | 0 |
| 2 | 15 | 1.5 | 41.0 | 8.5 | 1.6 | 15.3 | 25.4 | 74.6 | 175 | 62 | 3.20 | 0 |
| 3 | 15 | 1.3 | 38.0 | 5.2 | 1.7 | 16.5 | 23.4 | 76.6 | 208 | 62 | 3.02 | |
| 20 cc. 40 per cent egg albumin solution intravenously | | | | | | | | | | | | |
| 4 | 5 | 4.7 | 42.4 | 6.6 | 2.1 | 17.0 | 25.7 | 74.3 | 208 | 61 | 2.31 | |
| 5 | 5 | 4.4 | 50.8 | 9.0 | | | | | 217 | 63 | 2.68 | |
| 6 | 5 | 8.4 | 56.6 | 4.6 | 4.0 | 23.0 | 31.6 | 68.4 | 208 | 60 | 3.04 | + |
| 7 | 15 | 6.0 | 48.6 | 5.0 | 3.6 | 13.8 | 22.4 | 77.6 | 190 | 58 | 3.05 | + |
| 8 | 15 | 4.7 | 41.5 | 9.6 | 2.2 | 16.9 | 28.7 | 71.3 | 166 | 58 | 2.40 | |
| 9 | 15* | 2.6 | 42.6 | 7.0 | 1.6 | 16.9 | 25.5 | 74.5 | 158 | 60 | 2.22 | + |
| 10 | 15* | 2.7 | 47.7 | 7.5 | 2.0 | 18.1 | 27.6 | 72.4 | 151 | 62 | 2.17 | |
| 11 | 15* | 3.0 | 45.6 | 8.1 | 3.0 | 18.8 | 29.9 | 70.1 | 133 | 60 | 2.15 | ++ |
| 12 | 30 | 2.6 | 47.7 | 7.5 | 3.2 | 19.2 | 29.9 | 70.1 | 126 | 56 | 2.0 | |
| 13 | 30 | 1.7 | 49.5 | 7.4 | 2.5 | 13.0 | 32.9 | 67.1 | 166 | | 2.19 | ++ |
| 14 | 30 | 1.9 | 43.8 | 11.0 | 3.0 | 22.0 | 36.0 | 64.0 | 151 | | 2.07 | ++ |
| Plasma | | | | | | | | | | | | |
| | 10:15 | | | | | | | | 181 | | 2.07 | (?) |
| | 10:45 | | | | | | | | 144 | | 1.67 | Trace |
| | 11:45 | | | | | | | | 133 | | 1.67 | Trace |
| | 1:40 | | | | | | | | 112 | | | |

* Restless.

PROTOCOL 6

*No. 479; February 8, 1923; weight 9000*

| NUMBER | TIME | VOLUME | PROTEIN | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN | TOTAL FIBRIN AND GLOBULIN | ALBUMIN | SUGAR | $CO_2$ | PHOSPHATES |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10:15–10:30 | 6.9 | 33.7 | 3.8 | 4.7 | 20.8 | 29.3 | 70.7 | 79.3 | 61 | |
| 2 | 10:30–10:45 | 7.1 | 34.5 | 4.3 | 3.0 | 25.0 | 32.3 | 67.7 | 86.9 | 60 | 3.31 |
| 3 | 10:45–10:50 | 9.3 | Lost | 3.0 | | | | | 149 | 58 | 4.08 |
| 4 | 10:50–10:55 | 8.3 | 34.8 | 0 | 4.4 | 47.0 | 51.4 | 48.6 | | 62 | 3.30 |
| 5 | 10:55–11:00 | 5.6 | 44.4 | 0 | 3.5 | 36.0 | 40.5 | 60.0 | 170 | 56 | 4.0 |
| 6 | 11:00–11:15 | 20.4 | 49.2 | 0 | 2.8 | 25.7 | 28.5 | 71.5 | 204 | 58 | 3.13 |
| 7 | 11:15–11:30 | 17.9 | 49.2 | 0 | 2.2 | 21.1 | 23.3 | 76.6 | 170 | 60 | 3.1 |
| 8 | 11:30–12:00 | 22.3 | 39.0 | 0 | 1.8 | 30.0 | 31.8 | 68.2 | 176 | 58 | 3.08 |
| 9 | 12:00–12:30 | 18.0 | 39.7 | 6.3 | 2.7 | 36.7 | 45.7 | 54.3 | 144 | 56 | 3.13 |
| 10 | 12:30– 1:00 | 14.4 | 39.1 | 4.8 | 9.4 | 34.0 | 48.2 | 51.8 | 144 | 50 | 3.13 |
| 11 | 1:00– 2:00 | 30.0 | 40.5 | 3.6 | 8.0 | 29.3 | 40.9 | 59.1 | 121 | 54 | 2.99 |
| 12 | 2:00– 3:00 | 25.3 | 42.3 | 3.5 | 7.2 | 10.9 | 31.6 | 68.4 | 162 | 52 | 2.63 |
| Dog not nauseated. No blood in lymph, but lymph becomes noncoagulable | | | | | | | | | | | |
| Plasma | | | | | | | | | | | |
| 1 | 10:30.............. | | 60.0 | 6.0 | | 40.1 | 46.1 | 53.9 | 111 | | |
| 2 | 11:00.............. | | 57.0 | 6.3 | | 43.5 | 49.8 | 50.2 | | | |
| 3 | 11:30.............. | | 54.5 | 6.5 | | 41.5 | 48.0 | 52.0 | 200 | | |
| 4 | 12:30.............. | | 51.0 | 6.4 | | 63.7 | 70.1 | 29.9 | | | |

Animal killed at 3:00 p.m. No marked engorgement of splanchnic area. Liver section taken.

PROTOCOL 7

*No. 480; February 9, 1923; weight 11,000; anaphylaxis; iron dog*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | | REDS | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS | FIBRIN AND GLOBULIN | ALBUMIN | PHOSPHATE | SUGAR | IRON PER 100 cc. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *mgm.* | | | | | | | | *mgm.* |
| 1 | 10:45–11:00 | 8.4 | 2.8 | 0 | 32.0 | 6.2 | 4.8 | 27.0 | 38.0 | 62.0 | 3.19 | 174 | 0.012 |
| 2 | 11:00–11:15 | 5.4 | 1.8 | 0 | 30.3 | 5.0 | 10.6 | 24.3 | 39.9 | 60.1 | 3.72 | 171 | |
| 3 | 11:15–11:30 | 6.6 | 2.2 | 0 | 31.9 | 6.0 | 9.0 | 24.5 | 39.5 | 60.5 | 4.0 | 219 | |
| 20 cc. of a 40 per cent solution of fresh egg albumin injected intravenously | | | | | | | | | | | | | |
| 4 | 11:30–11:35 | 12.4 | 12.4 | 0 | 38.3 | 10.7 | 2.9 | 21.7 | 35.3 | 64.7 | 2.5 | 233 | 0.012 |
| 5 | 11:35–11:40 | 13.0 | 13.0 | +++ | 43.4 | 1.8 | 5.0 | 49.0 | 55.8 | 44.2 | 3.68 | 243 | 0.0134 |
| 6 | 11:40–11:45 | 9.6 | 9.6 | +++ | 40.4 | 1.0 | 9.0 | 34.7 | 44.7 | 55.3 | 3.65 | 225 | 0.0134 |
| 7 | 11:45–12:00 | 23.0 | 7.7 | ++ | 40.6 | 1.7 | 4.0 | 36.8 | 42.5 | 57.5 | 4.32 | 225 | 0.015 |
| 8 | 12:00–12:15 | 15.0 | 5.0 | + | 39.8 | 11.3 | 2.7 | 36.6 | 50.6 | 49.4 | 4.06 | 222 | 0.0131 |
| 9 | 12:15–12:30 | 16.0 | 5.3 | +++* | 35.2 | 11.3 | 1.7 | 33.0 | 46.0 | 54.0 | 3.84 | 189 | 0.0135 |
| 10 | 12:30–12:40 | 7.2 | 3.6 | +++* | 34.3 | 9.0 | 2.3 | 33.3 | 44.6 | 55.4 | 4.06 | 190 | 0.0126 |

* Restless. Animals became very restless at 12:15 and was taken from board at 12:40. The animal was evidently feeling well, strong, pulse full. No effect apparent as far as general condition was concerned. Had had a liquid stool at 12 noon.

Of the three animals which received colloidal iron only one showed red blood corpuscles in the lymph after the shocking dose (protocol 8). This dog also gave other evidence of decided shock such as transient diarrhea and increase in sugar and phosphates. This dog was very restless and was taken from the board one hour after injection. At this time the pulse, respiration and muscular strength showed the animal to be in excellent condition. The animal remained in good condition during the twenty-four hours he was kept under observation. The lymph of the dog described in protocol 5 was also examined for iron, which was present in all samples. There was no apparent increase during or after shock.

## DISCUSSION

Anaphylactic shock in the dog, as we interpret the findings in the lymph stream, consists primarily of an endothelial shock. In this shock that portion of the endothelium which is normally most permeable, that of the splanchnic area, is most concerned. As a result of a great stimulus, or of irritation, the permeability of the endothelium is suddenly increased, a variation which in fatal cases goes over to an irreversible condition of "fatigue." The sudden increase of permeability throws an enormous quantity of fluid into the hepatic lymphatics, the liver becomes distended, the capillaries disorganized and blood corpuscles appear in the lymph. The specific protein to which the cells have been sensitized can rapidly leave the capillaries and come in contact everywhere with the cells of the parenchyma, resulting in a primary stimulus manifested by an increase in fibrinogen, globulins, sugar and phosphates. Coincidently with the primary stimulus there has been a liberation of antithrombic substances as well as proteolytic enzymes; these now alter the protein picture so that only smaller molecular aggregates will appear in the lymph, but phosphates and sugar will continue to leave the injured cells. A second factor will appear if the dog lives long enough. The passive hyperemia, as induced above, produces more tissue injury resulting in a secondary shock, similar in character to that which we have described in experiments with intraportal oil injections.

The "endothelial blockade," such as we have produced with injections of sacchrated iron oxide, evidently modifies this picture to some extent. In the second series we have shown that while the control animal died, all three "protected" animals have survived, and only one of these showed marked evidences of alterations in the lymph. The effect of "protection" has become apparent in different ways. In some animals there has been no lymphagogue effect, in others the lymph remained free from blood corpuscles, in others the coagulation mechanism was not affected, in others the phosphate and sugar metabolism was decidedly altered.

From the experiments we believe that we are justified in the assumption that the shock of the endothelium is one of the primary factors in bringing about the acute symptoms of anaphylactic shock in the dog, and that this shock can be altered when the endothelium is altered, as in "endothelial blockade." The term "endothelial blockade" is one, however, which is perhaps misleading, implying an alteration in the endothelium whereby it becomes less permeable. This is by no means certain. Siegmund (3) has, for instance, found that the injection of various colloids seems to alter the endothelial cells so that they are more active in taking up dyes and pigments. A modification of the anaphylactic reaction such as we have observed might therefore be interpreted as due either (*a*) to a true blockade of the cells or due to (*b*) an increased activity of the endothelial elements involving perhaps a more rapid destruction of the antigen and a consequent protection of the parenchymal cells of the splanchnic area.

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# STUDIES ON ENDOTHELIAL PERMEABILITY. V

## THE EFFECT OF PEPTONE ON THE PERMEABILITY OF THE ENDOTHELIUM

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During the course of the studies on endothelial permeability made with thoracic duct fistulas we have had occasion to study the effect of varying doses of peptone injected intravenously. The effect of peptone has been studied intensively; usually relatively large doses have been injected and it has been found that there undoubtedly follows an increase in the rate at which colloids (hemolysins, agglutinins, antitoxins, foreign serum, etc.) pass through from the capillary bed to the lymph stream. Yet there seems some doubt whether we are dealing with an actual alteration in the permeability of the endothelium or are dealing purely with an increased intraportal pressure, as Abe (1) suggests. Starling inclines to the view that both an increase in intraportal pressure and an increased permeability of the endothelium must take place (2). If we deal with the purely mechanical factor of increased intraportal pressure we must assume that the lymph will not be altered in concentration, or if altered, the proteins will be at first diminished in amount, as we have demonstrated when we produce an intraportal blockade. If, on the other hand, we deal with alterations in permeability, then we might expect an increase in the concentration of proteins even when the intraportal pressure is little altered; we might also anticipate that following the primary stimulus the endothelial permeability will, if the dose be large, increase as fatigue sets in; if the dose has been small, it will diminish when the reversal of the state of the cell membrane takes place.

The following protocols illustrate our findings:

PROTOCOL 1

*No. 438*

| NUMBER | TIME | VOLUME PER 5 MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | SUGAR |
|---|---|---|---|---|---|
| | *minutes* | *cc.* | *mgm.* | *per cent* | |
| 1 | 15 | 1.0 | | | |
| 2 | 15 | 0.7 | 44.0 | 5.9 | 118 |
| 3 | 15 | 1.4 | | | |
| 4 | 15 | 1.5 | | | |
| 0.3 gram peptone intravenously | | | | | |
| 5 | 5 | 1.2 | | | |
| 6 | 5 | 1.7 | 49.2 | 6.0 | 108 |
| 7 | 5 | 2.4 | | | |
| 8 | 15 | 1.66 | 46.1 | 6.3 | 118 |
| 9 | 15 | 1.2 | 45.8 | 6.1 | 111 |
| 10 | 15 | 1.5 | | | |
| | | | 43.9 | 5.7 | 111 |
| 11 | 15 | 1.4 | | | |
| 12 | 15 | 1.4 | | | |
| | | | 42.3 | 5.4 | 125 |
| 13 | 15 | 1.2 | | | |
| 14 | 30 | 1.6 | 41.2 | 5.3 | 124 |
| 15 | 30 | 0.9 | 39.4 | 5.5 | 92 |

*Comment.* With this small dose (which is still much greater than that used therapeutically) only a slight lymphagogue effect can be noted, but the protein of the lymph is increased. This, however, diminishes continuously and within an hour and a half has already become less concentrated than before the injection. It later is diminished about nine per cent below the preinjection level. With these small doses of peptone there is no incoagulability of the lymph, indeed the fibrin is actually increased in amount to a slight extent. At the end of the experiment, protein, sugar and fibrin are all at a lower level than before the injection.

PROTOCOL 2

*No. 442; weight 7800; 50 mgm. peptone per kilo*

| NUMBER | TIME | VOLUME PER 5 MINUTES | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | SUGAR | BILE PIGMENT | HEMOGLOBIN | SUGAR IN BLOOD PLASMA |
|---|---|---|---|---|---|---|---|---|
| | *minutes* | *cc.* | *mgm.* | *per cent* | | | *per cent* | |
| 1 | 30 | 1.4 | 39.8 | 4.0 | 142 | | 0 | 98 |
| 400 mgm. peptone plus 15 cc. hemoglobin solution intravenously | | | | | | | | |
| 2 | 5 | 4.6 | 40.2 | 5.2 | 146 | | 5 | |
| | | | | | | | | 133 |
| 3 | 5 | 7.6 | 53.5 | 4.8 | 173 | | 15 | |
| 4 | 5 | 4.5 | | 4.4 | 176 | | 15 | |
| 5 | 5 | 3.6 | 47.7 | 4.4 | 176 | | | |
| 6 | 5 | 2.6* | | 4.4 | 173 | | 13 | |
| 7 | 30 | 1.9 | 42.5 | 5.8 | 115 | | | |
| | | | | | | | | 90 |
| 8 | 30 | 1.3 | 42.0 | 4.7 | 111 | | | |
| 9 | 30 | 1.5 | 42.0 | 4.7 | 111 | | | |
| | | | | | | | | 94 |
| 10 | 30 | 1.5 | 37.8 | 3.7 | 80 | Trace | | |
| 11 | 30 | 1.3 | 35.5 | 3.6 | 87 | Trace | | |
| | | | | | | | | 83 |
| 12 | 30 | 0.8 | 37.7 | 3.2 | 80 | Trace | | |

* Animal has chill.

*Comment.* In this experiment a larger dose of peptone was injected but it was given simultaneously with 15 cc. of a hemoglobin solution, so that the toxicity was modified. It will be noted that the hemoglobin makes its appearance quite promptly (within five minutes after intravenous injection; i. e., within the same time that crystalloids pass through from the blood stream to the lymph stream), striking evidence of the prompt increase in permeability.

The lymphagogue effect was somewhat more marked in this experiment, as was also the effect on the sugar mobilization. Here again the level of the protein, the fibrin, and the sugar was lowered at the end of the experiment.

When now we turn to a same dose given in a concentrated form (5 cc. of saline) the effects are quite different.

PROTOCOL 3

*No. 474; weight 7000; peptone 50 mgm. per kilo*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | SUGAR | $CO_2$ | PHOSPHATES | RESISTANCE |
|---|---|---|---|---|---|---|---|---|
| | *minutes* | *cc.* | *mgm.* | *per cent* | | | | |
| 1 | 15 | 1.3 | 34.5 | 5.7 | 151 | 68 | 432 | 5.25 |
| 2 | 15 | 1.0 | 36.1 | 2.7 | 145 | 58 | | 5.31 |
| 350 mgm. peptone intravenously | | | | | | | | |
| 3 | 5 | 2.9 | *46.2* | 0 | 148 | 55 | *5.64* | 5.05 |
| 4 | 5 | 3.5 | 42.2 | 0 | 152 | 55 | 3.83 | 5.09 |
| 5 | 5 | 3.1 | | 0 | 172 | ╱48 | | 4.85 |
| 6 | 15 | 2.36 | 40.0 | 0 | 200 ↙ | ←*38* | 3.33 | *4.71* |
| 7 | 15 | 2.66 | 35.1 | 0 | 144 | 58 | 3.73 | 4.92 |
| 8 | 15 | 1.85 | 36.55 | 7.2 | 148 | ╱48 | 3.41 | 5.05 |
| 9 | 15 | 1.6 | 42.5 | 8.2 | 155 ↙ | 56 | 3.76 | 5.05 |
| 10 | 15 | 1.9 | 41.7 | 8.5 | 118 | ╱44 | 4.16 | 5.07 |
| 11 | 30 | 1.8 | 42.1 | 9.5 | 129 ↙ | 54 | 4.16 | 4.98 |
| 12 | 30 | 1.3 | *45.1* | 8.2 | 124 | 50 | *4.80* | 4.92 |
| 13 | 60 | 1.15 | 44.7 | 7.4 | 118 | 53 | 4.16 | 5.09 |
| 14 | 60 | 0.9 | 42.3 | 7.0 | 118 | | 4.10 | |

*Comment.* In this experiment we must distinguish two phases of stimulation, the one induced by the primary injection of the peptone, the other a secondary effect due probably to liver injury and the resulting liberation of further toxic products from the liver. It is not to be excluded that this secondary effect may in part be due to the effects of the primary hyperaemia of the splanchnic area, the stasis in itself causing tissue asphyxia, injury, and a secondary liberation of toxic products. When we examine the effects on the concentration of the proteins of the lymph we find the primary increase following immediately on the peptone injection. This seems to be followed by a prompt recovery, with diminished permeability. (It is not to be excluded that the dilution of the lymph proteins observed here may not be due to the increased intraportal pressure, but we are inclined to the view that the cause is to be sought rather in a prompt reversal of the endothelial membrane.) There then follows the typical secondary rise in lymph protein beginning with sample 11 and continued during sample 12. Both the phosphate and resistance curves of the lymph plasma show alterations that are related

to this secondary rise. It will be noted that the phosphates increase immediately after the peptone injection, then diminish markedly, but a secondary rise occurs in sample 12. Maximal ionization takes place some time after the primary increase in phosphates, and a second period of lowered electrical resistance takes place in sample 12. There seems also a definite relation between the acidosis and the sugar excretion. A maximal sugar concentration is reached in tube 6 which also represents the lowest $CO_2$ reading. Rhythmic fluctuations then take place in the $CO_2$ and each time the lower level is reached an increase in sugar output follows in the next sample of lymph. In this animal the preinjection levels for total protein and fibrin had not been reëstablished at the end of the experiment.

PROTOCOL 4

*No. 439; weight 13000; peptone 50 mgm. per kilo*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | NON-PROTEIN NITROGEN | PROTEASE | BILE PIGMENT | FIBRIN | EUGLOBULIN AND PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | TOTAL FIBRINOGEN AND GLOBULINS | ALBUMIN |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *cc.* | *mgm.* | | | | *per cent* | | | | *per cent* |
| 1 | 10:30–11:00 | 2.6 | 39.4 | 0.15 | 0.05 | 0 | 2.0 | 29.0 | 27.0 | 58.0 | 42.0 |
| 650 mgm. Witte peptone injected intravenously | | | | | | | | | | | |
| 2 | 11:00–11:05 | 7.8 | 42.0 | 0.24 | 0.06 | 0 | 2.1 | 36.0 | 22.0 | 60.1 | 39.9 |
| 3 | 11:05–11:10 | 9.0* | 50.0 | 0.36 | 0.07 | 0 | 0 | 33.0 | 28.0 | 61.0 | 39.0 |
| 4 | 11:10–11:15 | 6.8 | 50.0 | 0.26 | 0.16 | 0 | 0 | 28.0 | 28.0 | 56.0 | 44.0 |
| 5 | 11:15–11:30 | 4.0 | 42.4 | 0.23 | 0.19 | Trace | 0 | 40.0 | 16.2 | 56.2 | 43.8 |
| 6 | 11:30–11:45 | 4.2 | 40.0 | 0.20 | 0.16 | Trace | 0 | 26.0 | 3.5 | 29.5 | 70.5 |
| 7 | 11:45–12:00 | 4.1 | 38.6 | 0.20 | 0.10 | + | 0 | 24.0 | 2.6 | 26.6 | 73.4 |
| 8 | 12:00–12:30 | 3.3 | 43.1 | 0.21 | 0 | + | 1.6 | 20.0 | 7.0 | 28.6 | 71.4 |
| 9 | 12:30– 1:00 | 3.3 | 43.7 | 0.26 | 0 | + | 3.0 | 22.0 | 6.4 | 31.4 | 68.6 |
| 10 | 1:30– 1:45 | 2.0 | 40.0 | 0.26 | 0 | +++ | 2.0 | 24.5 | 2.7 | 19.2 | 70.8 |

* Red blood corpuscles through at 11:08.

Plasma before injection

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | NON-PROTEIN NITROGEN | PROTEASE | BILE PIGMENT | FIBRIN | EUGLOBULIN AND PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | TOTAL FIBRINOGEN AND GLOBULINS | ALBUMIN |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11:05 | | 48.4 | | | 0 | | 33.0 | 8.6 | 41.6 | 58.4 |
| | 11:30 | | 48.4 | | | 0 | | 31.0 | 8.6 | 39.6 | 60.4 |
| | 12:30 | | 48.4 | | | 0 | | 28.0 | 10.0 | 38.0 | 62.0 |
| | 2:00 | | 45.0 | | | 0 | | 34.0 | 11.0 | 45.7 | 54.3 |
| | | | 42.6 | | | 0 | | | 35.0 | | |

In protocol 4 we illustrate the same dose, again followed the secondary rise in the protein of the lymph an hour after the injection. This corresponds with a similar increase in the non-protein nitrogen of the lymph. Protease, determined by chloroform digestion of the lymph proteins and expressed in terms of milligrams digestion per cubic centimeter is increased after the injection and remains high during the time that the lymph remains incoagulable. A marked alteration in the relation of the lymph proteins is manifested by a transient increase in globulins after the injection, followed by a marked increase in the albumins, this followed by an increase in the larger molecular aggregates (particularly fibrin) at the time of the secondary increase in lymph proteins. An analysis of the proteins of the blood plasma reveals the relatively great concentration of the lymph proteins in comparison thereto. In this experiment the preinjection level of the lymph proteins had been restored at the end of the experiment.

PROTOCOL 5

*No. 437; December 4, 1922; weight 6.9 kilos; peptone 0.1 gram per kilo*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | SUGAR | FIBRIN | EUGLOBULIN AND PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | ALBUMIN | BILE PIGMENT |
|---|---|---|---|---|---|---|---|---|---|
| | | *cc.* | *mgm.* | | | | *per cent* | *per cent* | |
| 1 | 10:15–11:00 | 0.9 | 39.42 | 141 | 3 | 23.0 | 12.1 | 61.9 | — — |
| 0.69 grams Witte peptone injected intravenously | | | | | | | | | |
| 2 | 11:00–11:05 | 0.4 | 43.7 | 132 | 0 | 22.2 | 18.7 | 59.1 | — — |
| 3 | 11:05–11:10 | 5.2 | 51.0 | 170 | 0 | 24.2 | 23.7 | 52.1 | — — |
| 4 | 11:10–11:15 | 4.4 | 51.9* | 232 | 0 | 23.0 | 24.0 | 53.0 | — — |
| 5 | 11:15–11:30 | 2.5 | 46.83 | 259 | 0 | 20.8 | 19.2 | 60.0 | — — |
| 6 | 11:30–11:45 | 2.3 | 45.5† | 50 | 0 | 18.8 | 20.4 | 61.8 | ++ |
| 7 | 11:45–12:00 | 1.3 | 47.0 | | 0 | | | | ++ |
| 8 | 12:45– 1:00 | 2.1 | 47.0‡ | 200 | 3.4 | 16.7 | 20.6 | 59.3 | ++ |
| 9 | 2:00– 2:30 | 0.8 | | 245 | 3.2 | | | | +++ |

* Red blood corpuscles appear.

† Slight hemolysis.

‡ Plasma is yellowish.

In protocol 5 we illustrate the effect of a larger dose of peptone (0.1 gram per kilo). The primary effect on the globulin-albumin ratio is quite apparent in the increase in the larger molecular aggregates, followed by a restoration to the original. Secondary rises are to be noted in the protein and sugar curves. In protocol 2 we showed that when hemoglobin is injected at the same time as peptone, the hemoglobin appears promptly in the lymph stream (within five minutes). If, however, we give the hemoglobin some time after the peptone injection, we find that the hemoglobin is retarded so that only a trace can be obtained at the end of the fifteen minute period.

Such an experiment is illustrated in protocol 6.

PROTOCOL 6

*No. 443; December 13, 1922; weight 7200; peptone*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | HEMOGLOBIN | BILE PIGMENT | SUGAR |
|---|---|---|---|---|---|
| | | *cc.* | *per cent* | | |
| 1 | 10:25–10:55 | 0.75 | | 0 | 160 |
| 350 mgm. peptone intravenously | | | | | |
| 2 | 10:55–11:00 | 1.2 | | 0 | 250 |
| 3 | 11:00–11:05 | 1.6 | | 0 | 400 |
| 4 | 11:05–11:10 | 1.1 | | 0 | 363 |
| 5 | 11:10–11:25 | 0.9 | | Positive | 298 |
| 6 | 11:25–11:40 | 0.8 | | Positive | 285 |
| 20 cc. hemoglobin solution intravenously | | | | | |
| 7 | 11:45–11:50 | | 0 | | |
| 8 | 11:50–11:55 | | 0 | | |
| 9 | 11:55–12:00 | | Trace | | |
| 10 | 12:00–12:15 | | 46 | | |
| 11 | 12:15–12:30 | | 46 | | |

## DISCUSSION

Analysis of the lymph of the thoracic duct following the intravenous injection of peptone indicates that we are dealing with changes primarily dependent on alterations of the endothelial permeability, associated with secondary alterations that

apparently take place in the liver parenchyma. Previous workers have shown that large molecular aggregates, hemolysins, agglutinins, foreign serum, precipitins, antitoxins, etc., pass more rapidly from the blood stream to the lymph after the injection of peptone, and Teague (3) suggested that the therapeutic effect of peptone in non-specific therapy was based largely on this fact. Osato has suggested that use could be made of the same fact whenever we wished to rapidly saturate the organism with an antitoxin, as in diphtheria.

The following interpretation of the experimental facts that we have presented in the preceding protocols seems warranted: The injection of small doses of peptone may be followed by little or no alteration of the intraportal pressure (occasionally a diminution with small doses) but there results a primary stimulation of the endothelium. This involves an increase in permeability, indicated by the increase in proteins, the relative increase in globulins, the rapid passage of hemoglobulin from the capillary to the lymph stream. Associated with this effect is a stimulation of the liver parenchyma (indicated by an increase in the fibrinogen, sugar, phosphate, diminution of the electrical resistance, etc.). These changes are rapidly reversible, the protein of the lymph diminishing, as does the phosphate, sugar, fibrin, etc., which are usually depressed below the preinjection level. The endothelium at this time is less permeable to hemoglobin.

When a larger dose of peptone is injected the evidences of the primary stimulation are similar in their general character, but are greater in extent. Non-protein nitrogen is increased, proteolytic enzymes are mobilized and the lymph becomes incoagulable. A fluctuating acidosis may be observed, the extent of which seems to have an important bearing of the degree of the sugar mobilization. These alterations are again reversible, the protein of the lymph diminishing, as does the sugar, the phosphate, the ratio of the globulins, etc.

Then a secondary rise takes place in which practically all the factors that we have studied take part. This second period is a result, we believe, of changes produced in the liver and

intestine. This may be the result of liver injury by the peptone. As a result secondary toxic products arise which are thrown into the circulation during the course of an hour or two after the injection and again intoxicate the organism (a vicious circle). It may, however, be the result of the partial portal blockade that is produced when the peptone injures the liver endothelium, causes edema and passive hyperasemia of the liver and, as a consequence, an acidosis; this to be followed by parenchymal degeneration, disintegration and the liberation of toxic substances into the general circulation. We incline to the belief that both factors play an important part in the production of an intoxication in peptone shock in dogs: the results of the experiments that we have carried out would seem to be in accord with this conception. This secondary alteration in the lymph which we have repeatedly observed might presumably be brought about, not by the injury of the liver parenchyma, but by a lowering of the portal blood pressure. A study of the portal blood pressure tracings recently published by Manwaring and his associates (5) in peptone shock of the dog makes this view rather improbable. Manwaring finds that a well marked primary rise in the intraportal blood pressure takes place, this followed by a continued fall during the hour after the injection, the final level being apparently somewhat lower than before the injection.

In view of the fact that peptone has found extensive employment in non-specific therapy both in this country and in European clinics, the effects that we have described with small doses are of some therapeutic interest. These doses are of course larger than those ordinarily used in clinical work but it is to be remembered that in the patient we frequently deal with an endothelial system already somewhat more permeable because of the stimulation of toxins arising from the infection; occasionally with an endothelial system that is actually in a state of fatigue. Injections in such individuals are naturally followed by more severe reactions than when given to normal persons and the clinical results are merely those that we expect to witness in severe shock. There can be no therapeutic warrant for such

employment. When, however, we deal with very small doses of peptone in individuals in whom we have no reason to believe that the endothelial system is irritated or fatigued, the clinical benefits that have been described seem logical. The small dose is followed by a transient stimulation and then a relatively lessened permeability. There will follow a relative increase in resistance to certain forms of intoxication, particularly those that we associate with sensitization in some form, such as urticaria, furunculosis, arthritis, migrane, angioneurotic edema, hay fever, etc. This is in agreement with the effects that have been described on nitrogen metabolism—a temporary increase followed by a diminution in the nitrogen excretion; on basal metabolism—a temporary increase followed by a diminution; on the water metabolism—a temporary increase in the water loss followed by a retention; on the weight curve—an increase in weight; on the temperature curve in cases of fever—an increase followed by a diminution that may be temporary or permanent; to mention but a few of the fundamental phenomena that seem to be associated with these reversible changes in the cell surfaces of the endothelium and the parenchymal cells of the major viscera.

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# STUDIES ON ENDOTHELIAL PERMEABILITY. VI

## ALTERATIONS OF THE THORACIC LYMPH FOLLOWING THE INJECTION OF OLD TUBERCULIN IN NORMAL AND TUBERCULOUS DOGS

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During the many years that tuberculin has been used in the therapy of tuberculosis, years in which it has had as enthusiastic approval as it has had unqualified condemnation by clinicians the world over, the basis of its use has centered about the idea that in some way tuberculin had value in increasing the "antibody resistance" to the infection or in desensitizing specifically against the toxic products of the infection. Just how this was induced has never been satisfactorily explained. Yet we do know definitely that if it has value in some cases, that value cannot be due to the antibody mechanism. Increase in antibody concentration in the blood need bear no relation whatever to either clinical improvement or regression.

Granted that therapeutic results may at times be observed, how are they brought about? We are of the opinion that we must seek the answer in a field seemingly quite remote from antibody reactions and one which has heretofore been little cultivated, namely in alterations in the endothelium, not only of the vascular system but the itinerant endothelium that plays so large a rôle in the tubercle itself, alterations that bring about change in the water balance of the tissues and the fluids permeating the tubercle.

Very shortly after the preparation of tuberculin Gärtner and and Römer, to whom we owe many interesting bacteriological observations, noted that the injection of tuberculin was followed

by some lymphagogue effect. The volume of the lymph stream and the solids of the lymph increased, the only difference from the lymphagogue effect of the first class as described by Heidenhain lay in the fact that the lymph seemed to clot more readily instead of becoming incoagulable. Given subcutaneously, there seemed little effect, but the number of lymphocytes increased in the lymph and the clotting increased.

Thirty years have elapsed since that time and few observations have been added that bear directly on the problem. May (2) as well as Reichmann (3) have noted that a diuretic effect may follow tuberculin injections and Escherich (4) as well as Saathoff (5) have ascribed the increase in weight following tuberculin injections as due to a change in the water balance of the tissues. To Meyer-Bisch (6) we owe a careful study which we should like to review very briefly.

Meyer-Bisch first calls attention to the daily physiological fluctuation of the water balance of the body. The fluctuation varies from 500 to 1500 grams, reaching a maximum in the evening and a normal level in the morning. Coincident with this there is a concentration of blood in the evening, the blood proteins in particular being considerably increased in amount. This daily variation occurs in the tuberculous individual as it does in the normal, with the exception that as cachexia supervenes, the blood proteins may be diminished. Meyer-Bisch then examined the relation in tuberculous individuals when tuberculin was given. The dose was from 0.5 to 1 mgm. O. T. and fever seldom followed, so that the changes that he has observed do not depend merely on febrile alterations. In 32 cases studied he noted that in 70 per cent an alteration of the normal daily alteration of the water balance was induced. The changes that he describes are the following:

*a.* In the first group the patient lost weight, the blood became more concentrated after the injections and this change persisted for a considerable period of time.

*b.* In the second group the blood at first became more dilute and the patient gained weight for a short period of time, then the reverse changes took place.

*c.* In the third group the blood became more dilute immediately after the injection and the patient increased in weight. This increase persisted.

That these effects were specific for the tuberculin seemed evident from the fact that intramuscular milk injections were not

PROTOCOL 1

*No. 446; December 18, 1922; weight 7800 Kilos; tuberculin (O. T. 1 cc).*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS I AND II | TOTAL FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | BILE | PHOSPHATE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | | *per cent* | | | | | | |
| 1 | 10:30–10:45 | 2.3 | 30.1 | 5.5 | 13.4 | 1.3 | 20.2 | 79.8 | 153 | 0 | 4.5 |
| 2 | 10:45–11:00 | 2.5 | 29.7 | 6.3 | 15.3 | 4.5 | 26.1 | 73.9 | 153 | 0 | 3.8 |
| 1 cc. O. T. intravenously | | | | | | | | | | | |
| 3 | 11:00–11:05 | 3.5 | 29.3 | 5.8 | 18.0 | 3.4 | 27.2 | 72.8 | 153 | 0 | 11.0 |
| 4 | 11:05–11:10 | 3.4 | 26.15 | 2.8 | 17.6 | 5.5 | 25.9 | 74.1 | 153 | 0 | 5.0 |
| 5 | 11:10–11:15 | 2.7 | 30.86 | 5.8 | 28.6 | 1.4 | 35.8 | 64.2 | 181 | 0 | 5.0 |
| 6 | 11:15–11:30 | 2.6 | 27.20 | 5.8 | 20.6 | 4.9 | 30.4 | 69.6 | | 0 | 5.0 |
| 7 | 11:30–11:45 | 2.6 | 30.8 | 5.2 | 14.6 | 3.0 | 22.8 | 77.2 | 160 | 0 | 4.15 |
| 8 | 11:45–12:00 | 1.7 | 31.37 | 4.2 | 14.6 | 5.9 | 24.7 | 75.3 | 135 | 0 | 4.5 |
| 9 | 12:00–12:15 | 1.9 | 31.63 | 5.3 | 5.7 | 14.4 | 25.4 | 74.6 | 119 | | 4.0 |
| 10 | 12:15–12:30 | 1.6 | | | | | | | | | |
| 11 | 12:30–12:45 | 1.4 | 32.75 | 5.5 | 8.3 | 10.3 | 24.1 | 75.9 | 97 | 0 | 4.25 |
| 12 | 12:45– 1:00 | 1.5 | | | | | | | | | |
| 13 | 1:00– 1:15 | 1.7 | 30.88 | 5.4 | 8.4 | 14.0 | 27.8 | 72.2 | 122 | 0 | 5.9 |
| 14 | 1:15– 1:30 | 1.2 | | | | | | | | | |
| 15 | 1:30– 2:00 | 1.1 | 31.07 | 5.0 | 9.0 | 10.9 | 24.9 | 75.1 | 118 | 0 | 4.7 |
| 16 | 2:00– 3:00 | 0.96 | 33.3 | 4.5 | 3.7 | 16.0 | 24.9 | 75.1 | 95 | Trace | 5.3 |
| 17 | 3:00– 4:00 | 0.97 | 32.1 | 3.7 | 3.9 | 17.4 | 25.0 | 75.0 | | Trace | 5.0 |

followed by the same results. He noted that other lymphagogues, among them arsenic, might have the same effect as tuberculin when given in small doses. These, however, had an effect on the normal individual as well as on the tuberculous. We are ob-

viously dealing here with an alteration of the endothelium of the capillaries and with the power of the cells and intercellular colloids of the body to hold water.

This Meyer-Bisch has recognized and he has carried out a number of experiments with lymph fistulas in dogs to illustrate the changes. He found that if he injected a small dose of salt or sugar into the blood stream and then studied the protein concentration, and the rate of flow, as well as the salt concentration, two definite alterations would invariably be found. A primary lymphagogue effect with an increase in the amount of protein contained in the lymph, this to be followed by a final reduction in the rate of flow and a diminution in the concentration of protein. This latter effect persisted for a considerable length of time. He concluded that both with tuberculin injections as with these small injections of salt and sugar the endothelium was so altered that it had become less permeable.

Inasmuch as this phase of the effect of tuberculin has heretofore received but little attention we have carried out a number of experiments and will illustrate the results with a series of typical protocols. In the first experiment is shown the effect of a large dose of tuberculin (1 cc. O. T.) when intravenously injected in a normal dog (No. 446).

*Comment.* A number of rather striking alterations will be observed. A lymphagogue effect follows immediately after the injection, without alteration in the protein content of the lymph, except a moderate secondary rise noted in samples 11 and 12, taken about an hour and a half after the injection. A curious reversal in the proportion of euglobulin to the pseudoglobulins takes place with tubes 9 and 10, a change which persists during the course of the experiment. Lymph sugar, after the initial increase, shows a secondary rise in samples 13 and 14, corresponding with an increase in phosphates. The very great increase noted immediately after the injection of the tuberculin in the phosphate curve is in part accounted for by the phosphates injected with the tuberculin. (One cubic centimeter contains approximately 40 mgm. inorganic phosphate.)

In the following two experiments a smaller dose was used and inasmuch as the results differ in some details we show both protocols.

*Comment.* A slight lymphagogue effect, reaching a maximum at the end of fifteen minutes, instead of immediately after the injection as with the larger dose. The protein of the lymph showing only minor alterations, with, however, a gradual reduction of fibrin as well as euglobulin. There is some evidence of a secondary effect in that the total fibrin as well as globulin is increased in sample 10, at the same time that the lymph sugar shows a final increase.

PROTOCOL 2

*No. 448; December 27, 1922; weight 8200; Normal O. T.*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS I AND II | FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | BILE PIGMENT | PHOSPHATE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | | | *per cent* | | | |
| 1 | 10:15–10:30 | 2.6 | 36.1 | 8.4 | 10.0 | 23.8 | 42.2 | 57.8 | 118 | 0 | 8.0 |
| 2 | 10:30–10:45 | 1.7 | 30.9 | 5.8 | 10.6 | 25.8 | 42.2 | 57.8 | 128 | 0 | 8.5 |
| 3 | 10:45–10:50 | 1.3 | 30.77 | 7.0 | 10.6 | 30.2 | 47.8 | 52.2 | 150 | 0 | 8.5 |
| | | | | | 1 cc. O. T. 1:100 dilution | | | | | | |
| 4 | 10:50–10:55 | 1.48 | 32.27 | 6.1 | 10.0 | 33.0 | 49.1 | 50.9 | 180 | 0 | 7.7 |
| 5 | 10:55–11:00 | 1.9 | | | | | 49.5 | | | | |
| 6 | 11:00–11:05 | 3.0 | 30.43 | 6.0 | 11.6 | 32.9 | | 50.5 | 145 | 0 | 7.9 |
| | | | | | | | 48.0 | | | | |
| 7 | 11:05–11:20 | 2.5 | 31.5 | 6.3 | 10.6 | 31.1 | | 52.0 | 140 | 0 | 7.3 |
| | | | | | | | 50.9 | | | | |
| 8 | 11:20–11:35 | 1.9 | 30.8 | 9.4 | 9.4 | 34.7 | | 49.1 | 125 | 0 | 8.5 |
| | | | | | | | 44.7 | | | | |
| 9 | 11:35–11:50 | 2.4 | 31.4 | 7.1 | 7.1 | 31.3 | | 55.3 | 140 | 0 | 7.9 |
| 10 | 11:50–12:05 | 1.4 | 29.4 | 7.1 | 7.1 | 36.3 | 49.9 | 50.1 | 158 | 0 | 8.0 |
| 11 | 12:05–12:35 | 1.3 | 28.7 | 5.5 | 7.0 | 32.4 | 44.9 | 55.1 | 131 | 0 | 8.0 |
| 12 | 12:35– 1:05 | 1.9 | 30.57 | 5.1 | 6.0 | 33.1 | 44.2 | 55.8 | 131 | 0 | 7.6 |

*Comment.* This animal responded with little alteration in the rate of flow and in general a reduction in the amount of lymph protein, except for a transient rise in samples 8 and 9. Just before this took place an increase in fibrin, euglobulin and pseudoglobulins was to be observed. In this experiment we find a well defined initial reduction in the lymph sugar, with an equally well defined and continued increase in the phosphates. When hemo-

PROTOCOL 3

*No. 447; December 20, 1922; weight 9200*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS III | FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | BILE PIGMENT | PHOSPHATE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | *per cent* | | *per cent* | | | |
| 1 | 9:45–10:00 | 1.6 | 41.5 | 3.6 | 0.5 | 23.5 | 27.6 | 72.4 | 151 | 0 | 4.8 |
| 2 | 10:00–10:15 | 1.2 | | | | | | | | | |
| | | | 47.6 | 2.5 | 0.5 | 21.0 | 24.0 | 76.0 | 130 | 0 | 4.7 |
| 3 | 10:15–10:30 | 1.3 | | | | | | | | | |
| O. T. 5 cc. 1:500 | | | | | | | | | | | |
| 4 | 10:30–10:35 | 1.45 | 45.1 | 2.8 | | | | | 58 | 0 | 5.3 |
| 5 | 10:35–10:40 | 1.4 | 45.9 | 2.8 | | | | | 58 | 0 | 5.2 |
| 6 | 10:40–10:45 | 1.4 | 42.7 | 2.5 | | | | | 55 | 0 | 5.3 |
| 7 | 10:45–11:00 | 1.2 | 40.7 | 3.1 | 1.6 | 26.0 | 29.7 | 70.3 | 74 | 0 | 6.3 |
| 8 | 11:00–11:15 | 1.6 | 44.7 | 3.1 | 3.1 | 17.3 | 23.4 | 76.6 | 100 | 0 | 5.7 |
| 9 | 11:15–11:45 | 0.96 | 44.8 | 2.5 | 1.1 | 16.0 | 19.6 | 80.4 | 129 | + | 5.3 |
| 10 | 11:45–12:15 | 0.85 | 42.8 | 2.7 | 0.7 | 18.7 | 22.1 | 77.9 | 111 | + | 5.3 |
| 11 | 12:15–12:45 | 1.0 | 42.0 | 2.7 | 0.7 | 18.4 | 21.8 | 78.2 | 151 | + | 6.7 |
| 12 | 12:45– 1:15 | 0.87 | 40.1 | 2.7 | 2.4 | 20.3 | 25.4 | 74.6 | 127 | + | 6.3 |

After completion Hb was injected. Appearance delayed in lymph.

globin was injected intravenously at the end of the experiment, its appearance in the lymph was retarded.

This experiment, in which the injection of O. T. 1:10 was followed by little lymphagogue effect, reveals in general a reduction of the concentration of lymph proteins, of sugar and of phosphates (after an initial increase). Hemoglobin injected an hour after the injection of the tuberculin was markedly delayed in appearance in the lymph stream, the first trace being apparent after twenty-four minutes.

### ANIMAL WITH SPONTANEOUSLY HEALED TUBERCULOSIS

The following experiment offers an interesting contrast from the preceding ones in that the injection of the tuberculin was followed by a much more marked reaction.

PROTOCOL 4

*No. 450; December 29, 1922; weight 9200, O.T.*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULINS I AND II | FIBRIN AND GLOBULINS | ALBUMIN | SUGAR | PHOSPHATE | HEMOGLOBULINS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | | | *per cent* | | | |
| 1 | 10:07–10:15 | 3.15 | 38.9 | 5.0 | 4.3 | 16.0 | 25.3 | 74.7 | 145 | | |
| 2 | 10:15–10:30 | 2.1 | 38.6 | 4.1 | 3.3 | 18.4 | 25.8 | 74.2 | 125 | 5.6 | |
| 1 cc. O. T. 1:10 intravenously | | | | | | | | | | | |
| 3 | 10:30–10:35 | 1.9 | 34.3 | 5.3 | 3.8 | 18.4 | 27.5 | 72.5 | 125 | 8.0 | |
| 4 | 10:35–10:40 | 2.4 | 36.1 | 8.6 | 3.8 | 18.2 | 30.6 | 69.4 | 115 | 5.4 | |
| 5 | 10:40–10:45 | 2.7 | 34.3 | 3.8 | 3.8 | 20.6 | 28.2 | 71.8 | 113 | 5.6 | |
| 6 | 10:45–11:00 | 2.3 | 35.9 | 0.6 | 5.2 | 16.6 | 22.4 | 77.6 | 125 | 5.3 | |
| 7 | 11:00–11:15 | 2.2 | 36.3 | 3.9 | 4.2 | 18.2 | 28.3 | 71.7 | 122 | 5.2 | |
| 8 | 11:15–11:30 | 2.0 | 37.8 | 0 | 6.7 | 17.8 | 24.5 | 75.5 | 117 | 5.5 | |
| 20 cc. Hb solution intravenously | | | | | | | | | | | |
| 9 | 11:30–11:35 | | | | | | | | | | |
| 10 | 11:35–11:40 | 1.4 | 38.3 | 3.4 | 8.2 | 19.7 | 31.3 | 68.7 | 133 | 6.2 | |
| 11 | 11:40–11:45 | | | | | | | | | | |
| 12 | 11:45–11:50 | | | | | | | | | | |
| 13 | 11:50–11:55 | 1.6 | 36.4 | 1.6 | 9.2 | 16.5 | 27.3 | 72.7 | 124 | 5.6 | 2.5 |
| 14 | 11:55–12:00* | | | | | | | | | | |
| 15 | 12:00–12:05 | | | | | | | | | | |
| 16 | 12:05–12:10 | 1.0 | 35.8 | 0.4 | 7.5 | 16.7 | 24.6 | 75.4 | 89 | 6.2 | 8.5 |
| 17 | 12:30–12:15 | | | | | | | | | | |
| 18 | 12:15–12:30 | 1.3 | 37.5 | 1.3 | 6.0 | 16.7 | 24.0 | 76.0 | 89 | 6.3 | 10.0 |
| 19 | 12:30–12:45 | 1.7 | 38.1 | 3.0 | 1.8 | 18.0 | 22.8 | 77.2 | 96 | 6.0 | 18.5 |

* Hemoglobin in this sample.

*Comment.* In this animal, presumably normal, we injected 1 cc. of old tuberculin in 20 cc. of hemoglobin solution. The injection, it will be noted, was followed by a decided lymphagogue effect which persisted for approximately two hours and was accompanied by an increase in the lymph proteins. This latter

PROTOCOL 5

*No. 465; January 11, 1923; weight 135; tuberculin and hemoglobin*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN EUGLOBULINS | PSEUDOGLOBULINS I AND II | TOTAL GLOBULINS AND FIBRIN | ALBUMIN | PROTEASE | NON-PROTEIN NITROGEN | HEMOGLOBIN | SUGAR | $CO_2$ | PHOSPHATE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | | | | | | | *per cent* | | | |
| 1 | 12:00–12:15 | 0.8 | | | | | | | | | 91 | 49.5 | |
| 2 | 12:15–12:30 | 1.2 | 55.6 | 1.53 | 22.8 | 24.33 | 75.67 | 0.29 | 10.0 | | 95 | 62.6 | 5.46 |
| 3 | 12:30–12:35 | 1.6* | | | | | | | | | 97 | 74.2 | |
| 4 | 12:35–12:40 | 5.1 | 57.12 | 4.6 | 21.0 | 25.6 | 74.4 | | | 26 | 101 | 63.6 | 6.40 |
| 5 | 12:40–12:45 | 5.2 | 60.0 | 3.6 | 20.1 | 23.7 | 76.3 | 0.53 | 10.0 | 35 | 125 | 65.3 | 6.40 |
| 6 | 12:45–12:50 | 5.1 | 57.6 | 3.3 | 20.0 | 23.3 | 76.7 | | | 32 | 138 | 66.5 | 6.84 |
| 7 | 12:50–12:55 | 3.5 | 57.12 | 4.5 | 19.2 | 23.7 | 76.3 | | | 37 | 133 | 52.8 | 6.32 |
| 8 | 12:55– 1:00 | 3.0 | | | | | | 0.30 | 24.5 | | 139 | 64.3 | |
| 9 | 1:00– 1:05 | 3.4 | 53.1 | 4.2 | 18.8 | 23.0 | 77.0 | | | 38 | 126 | 62.6 | 6.74 |
| 10 | 1:05– 1:15 | 2.3 | | | | | | | | | 121 | 73.9 | |
| 11 | 1:15– 1:30 | 2.3 | 53.1 | 4.7 | 17.3 | 22.0 | 78.0 | 0.32 | 19.6 | 30 | 116 | 68.1 | 6.66 |
| 12 | 1:30– 2:00 | 2.1 | 49.16 | 5.3 | 19.5 | 24.8 | 75.2 | | | 28 | 111 | 69.4 | 6.76 |
| 13 | 2:00– 2:30 | 2.3 | 51.0 | 3.5 | 20.4 | 23.9 | 76.1 | | | 32 | 98 | 64.5 | 7.46 |
| 14 | 2:30– 3:00 | 1.8 | 49.2 | 3.2 | 21.0 | 24.2 | 75.8 | | | | 98 | 66.4 | 6.88 |
| 15 | 3:00– 3:30 | 1.4 | 52.4 | 3.0 | 26.5 | 29.5 | 70.5 | | | 40 | 96 | | 6.84 |

* Twenty cc. hemoglobin solution with 1 cc. O. T. injected during this time. Blood corpuscles appeared in sample 4 and continued.

increase was of shorter duration, the final amount of protein in the lymph being under the preinjection level. Hemoglobin appeared in concentrated amount in the lymph within five minutes after the injection. The lymph was collected under oil and exalated, so that separate fibrin determinations have not been made

in this experiment, but the precipitate with 14 per cent sodium sulphate shows a marked increase after the injection. An increase in proteolytic enzyme, in phosphate, in sugar and in non-protein nitrogen were all observed. The increase in the larger protein aggregates apart from those precipitated by 14 per cent sodium sulphate did not take place until the end of the experiment. The primary increase in permeability is of course evident and we believe that this alteration persisted during the course of the experiment because of the long continued lymphagogue effect, as well as the persistent increase in phosphates and in the larger protein molecules.

The carbon-dioxide, it will be noted, revealed no evidence of an acidosis.

At the autopsy, controlled by later histological examination, the following evidences of healed tuberculosis were present. In the lungs two gelatinous scars, each approximately 0.5 cc. in length, extending from the pleura into the parenchyma of the lung, were present. The retrosternal and mediastinal lymph glands were somewhat enlarged, the tracheobronchial lymph glands enlarged and contained, apart from masses of coal pigment, minute whitish scars. The right kidney contained a deep, depressed scar, approximately 2 by 0.5 cm. in size, extending from the cortex to the pelvus, through the parenchyma. In the liver a few minute, yellowish miliary scars were observed scattered in the perivascular intralobular connective tissue. The picture was quite typical of that which we frequently see in the healed tuberculosis after inoculation of tubercle bacilli in dogs.

## EFFECTS OF TUBERCULIN IN INOCULATED ANIMALS

When we now study the effect in the tuberculous animal the changes that take place evidently become much more complex in their interrelation. The results in almost every animal studied have varied in some detail: we therefore present a series of protocols. The animals all received a mixed intravenous injection of virulent bovine and human tubercle bacilli from three to four weeks before the tuberculin injection.

PROTOCOL 6

*Dog 411; October 2, 1922; weight 800 kilos; inoculated September 9, 1922*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NUMBER | TIME | VOLUME PER FIVE MINUTES | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | FIBRIN AND GLOBULINS | ALBUMIN |
| | | | *mgm.* | *per cent* | *per cent* | | | | *per cent* |
| 1 | 11:45–12:00 | 4.3 | 42.8 | 4.2 | 1.1 | 10.0 | 2.4 | 25.7 | 74.3 |
| 2 | 12:00–12:15 | 4.0 | 41.6 | 3.8 | 0.1 | 20.0 | 2.8 | 26.7 | 73.3 |
| 3 | 12:15–12:30 | 3.5 | | | | | | | |
| 4 | 12:30–12:45 | 3.1 | 43.5 | 3.9 | 0.1 | 20.0 | 2.8 | 26.8 | 73.2 |
| 5 | 12:45– 1:00 | 3.4 | | | | | | | |
| 0.5 cc. O. T. injected intravenously | | | | | | | | | |
| 6 | 1:00– 1:15 | 5.0 | 48.62 | 3.8 | 0.2 | 20.6 | 0 | 24.6 | 75.4 |
| 7 | 1:15– 1:30 | 3.3 | 48.6 | 3.9 | 0.3 | 20.6 | 1.0 | 25.8 | 74.2 |
| 8 | 1:30– 1:45 | 3.0 | 39.9 | 3.3 | 0.3 | 21.5 | 1.7 | 26.8 | 73.2 |
| 9 | 1:45– 2:00 | 2.9 | | | | | | | |
| 10 | 2:00– 2:15 | 2.7 | 37.6 | 3.2 | 0.4 | 21.2 | 0.8 | 25.6 | 74.4 |
| 11 | 2:15– 2:30 | 2.7 | | | | | | | |
| 12 | 2:30– 2:45 | 2.7 | 45.08 | 3.1 | 0.2 | 18.4 | 1.8 | 23.5 | 76.5 |
| 13 | 2:45– 3:00 | 2.5 | | | | | | | |
| 14 | 3:00– 3:15 | 2.4 | 44.9 | 2.9 | 0.3 | 18.4 | 0 | 21.6 | 78.4 |
| 15 | 3:15– 3:30 | 1.8 | | | | | | | |
| 16 | 3:30– 3:45 | 2.0 | 45.0 | 2.8 | 0.35 | 18.4 | 2.0 | 23.5 | 76.5 |
| 17 | 3:45– 4:00 | 2.0 | | | | | | | |
| 18 | 4:30– 5:00 | 1.4 | 51.8 | 3.4 | 0.2 | 16.8 | 1.5 | 21.9 | 78.1 |
| Next morning | | | | | | | | | |
| 19 | 9:00– 9:30 | 0.6 | 51.9 | 3.2 | 0.4 | 18.3 | 3.1 | 25.0 | 75.0 |
| 20 | 9:30–10:00 | 0.55 | | | | | | | |
| Plasma | | | | | | | | | |
| | | | 67.34 | 1.8 | | 18.6 | 5.6 | 26.0 | 74.0 |

In this animal receiving a relatively large dose of tuberculin only a transient lymphagogue effect was apparent, accompanied by a temporary increase in lymph protein. A reversal then took place and the protein became less in amount. An hour and one half later we again find a reversal and this time the increase in

PROTOCOL 7

*Dog 412; October 4, 1922; weight 7, 600; inoculated September 8, 1922*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | TOTAL PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDO-GLOBULIN I | PSEUDO-GLOBULIN II | FIBRIN AND GLOBULINS | ALBUMIN |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| 1 | 10:15–10:30 | 5.9 | 63.8 | 5.9 | 0 | 20.0 | 0 | 25.9 | 74.1 |
| 2 | 10:30–10:45 | 4.3 | | | | | | | |
| 3 | 10:45–11:00 | 4.6 | 60.4 | 6.6 | 0 | 19.0 | 0 | 25.6 | 74.4 |
| 4 | 11:00–11:15 | 6.3 | | | | | | | |
| 5 | 11:15–11:30 | 5.5 | 60.6 | 6.6 | 0 | 17.0 | 6.0 | 31.0 | 69.0 |
| 6 | 12:30–12:45 | 9.8 | 59.8 | 5.7 | 0 | 16.3 | 4.6 | 26.6 | 73.4 |
| 1 cc. O. T. 1:4 dilution | | | | | | | | | |
| 7 | 12:45– 1:00 | 4.2 | 59.8 | 5.7 | 0 | 15.6 | 5.3 | 26.6 | 73.4 |
| 8 | 1:00– 1:15 | 2.8 | 53.5 | 6.0 | 0 | 16.0 | 7.0 | 29.0 | 71.0 |
| 9 | 1:15– 1:30 | 2.6 | | | | | | | |
| 10 | 1:30– 1:45 | 2.5 | 53.5 | 5.6 | 0 | 16.5 | 4.5 | 26.6 | 73.4 |
| 11 | 1:45– 2:00 | 2.5 | | | | | | | |
| 12 | 2:00– 2:15 | 1.9 | 58.6 | 5.0 | 0 | 16.4 | 2.4 | 23.6 | 76.4 |
| 13 | 2:15– 2:30 | 1.0 | | | | | | | |
| 14 | 2:30– 2:45 | 1.4 | 45.0 | 7.0 | 0 | 21.3 | 3.5 | 31.8 | 68.2 |
| 15 | 2:45– 3:00 | 2.7 | | | | | | | |

Animal died at 3 p.m. Red blood corpuscles appeared in the lymph after the tuberculin injection.

the amount of protein in the lymph persists into the samples collected the following morning. The alterations in the fibrin, globulins, and albumin seem relatively negligible. At autopsy a miliary tuberculosis, involving chiefly the liver, was observed.

This animal was very restless, a fact that may account for the alteration in the pseudoglobulin II fraction observed in samples 5 and 6. The animal became quiet and a dose of 1 cc. of 1:4 O. T. was injected at 12.45. This was followed by some lymphagogue effect, the appearance of red blood corpuscles in the lymph stream,

PROTOCOL 8

*Dog 413, October 7, 1922; weight 6100; inoculated September 8, 1922*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | TOTAL GLOBULIN AND FIBRIN | NON-PROTEIN NITROGEN | ALBUMIN |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | *per cent* | *per cent* | | *per cent* | |
| 1<br>2 | 9:30– 9:45<br>9:45–10:00 | 1.6<br>2.4 | 45.1 | 4.3 | 1.0 | 40.0 | 0 | 45.3 | 0.6 | 54.1 |
| 3<br>4 | 10:00–10:15<br>10:15–10:30 | 2.7<br>2.3 | 43.9 | 4.7 | 0.5 | 37.5 | 0 | 42.7 | 0.7 | 56.6 |
| 1 cc. O. T. 1:200 | | | | | | | | | | |
| 5<br>6 | 10:30–10:45<br>10:45–11:00 | 3.7<br>3.3 | 44.0 | 5.0 | 0.4 | 33.2 | 0 | 38.6 | 0.7 | 60.7 |
| 7<br>8 | 11:00–11:15<br>11:15–11:30 | 3.7<br>2.9 | 42.1 | 5.0 | 0.2 | 32.5 | 0 | 37.7 | 0.7 | 61.6 |
| 9<br>10 | 11:30–11:45<br>11:45–12:00 | 2.7<br>2.1 | 37.57 | 5.0 | 0.1 | 33.2 | 0 | 38.3 | 4.4 | 57.3 |
| 11 | 12:00–12:15 | 2.1 | 31.0 | 6.0 | 0.4 | 37.7 | 0 | 44.7 | 6.0 | 49.9 |
| 12 | 2:00– 2:15 | 1.3 | 30.57 | 5.0 | 0.4 | 39.0 | 0 | 44.4 | 6.0 | 49.6 |

Animal killed at 2.15 because restless.

but with a reduction in the amount of lymph proteins. This may have its explanation in the fact that the concentration of the lymph proteins in the preinjection period was already great, being practically that which we find in the normal plasma. The animal died at 3 p.m. Autopsy findings as in dog 411.

The dose here was much smaller (1 cc. 1:200). A lymphagogue effect was apparent, but the animal was restless after the injec-

tion so that the result must be considered with some reservation. Lymph proteins diminished decidedly while the fibrin increased. A striking alteration is to be observed in the amount of non-protein

PROTOCOL 9

*No. 425; November 13, 1922; weight 8300 kilos; inoculated October 8, 1922*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | TOTAL PROTEIN PER CUBIC CENTIMETER | PLASMA SUGAR | FIBRIN | EUGLOBULINS | PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | ALBUMIN | BILE PIGMENT |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| 1 | 10:30–10:45 | 3.0 | 36.0 | 133 | 3.0 | 4.0 | 50.5 | 0 | 42.5 | 0 |
| 2 | 10:45–11:00 | 3.1 | | | | | | | | |
| Injected 1 cc. of 1:10,000 O. T. | | | | | | | | | | |
| 3 | 11:00–11:15 | 3.8 | 33.4 | 115 | 3.2 | 11.1 | 37.5 | 0 | 48.5 | |
| 4 | 11:15–11:30 | 3.4 | 33.5 | | 3.0 | 10.6 | 38.2 | 0 | 48.2 | |
| 5 | 11:45–12:00 | 5.2 | 33.0 | 133 | 3.3 | 8.2 | 31.2 | 3.3 | 54.0 | 0 |
| 6 | 12:00–12:15 | 3.3 | | | | | | | | |
| 7 | 12:15–12:30 | 2.9 | 32.2 | 133 | | 7.8 | 32.6 | 8.4 | 49.2 | |
| 8 | 12:30–12:45 | 3.6 | | | | | | | | |
| 9 | 12:45– 1:00 | 2.1 | 29.6 | 133 | 4.7 | 9.0 | 40.0 | 2.0 | 44.3 | 0 |
| 10 | 1:00– 1:15 | 3.1 | | | | | | | | |
| 11 | 1:15– 1:30 | 3.0 | 34.0 | | 5.8 | 11.1 | 35.6 | 0 | 47.5 | |
| 12 | 4:15– 4:45 | 1.3 | 33.7 | | 3.5 | 13.0 | 39.4 | 0 | 44.1 | |
| Next morning | | | | | | | | | | |
| 13 | 9:30– 9:45 | 4.5 | 35.2 | 133 | 4.2 | 15.0 | 28.2 | 9.6 | 43.0 | 0 |
| 14 | 9:45–10:00 | | | | | | | | | |
| Plasma | | | | | | | | | | |
| | | | 64.4 | 133 | | 18 | 22.2 | 7.4 | 41.6 | 0 |

nitrogen which was determined in this experiment. Autopsy findings as in 411.

In the following protocols we illustrate the effects of smaller doses of tuberculin.

PROTOCOL 10

*No. 427; November 16, 1922; weight 7800; inoculated October 8, 1922*

| NUMBER | TIME | VOLUME PER FIVE MINUTES | SUGAR IN LYMPH | PROTEIN PER CUBIC CENTIMETER | FIBRIN | EUGLOBULIN AND PSEUDOGLOBULIN I | PSEUDOGLOBULIN II | ALBUMIN |
|---|---|---|---|---|---|---|---|---|
| | *minutes* | | | *mgm.* | *per cent* | | *per cent* | |
| 1 | 10 | 5.6 | 117 | 48.4 | 6.0 | 36 | 30 | 28.0 |
| 2 | 10 | 4.25 | | | | | | |
| 3 | 10 | 4.8 | | | | | | |
| 4 | 10 | 4.5 | | | | | | |
| 1 cc. 1:100,000 O. T. injected | | | | | | | | |
| 5 | 5 | 4.8 | 146 | 46.3 | 5.6 | 38 | 34 | 22.4 |
| 6 | 5 | 4.4 | | | | | | |
| 7 | 10 | 3.7 | | | | | | |
| 8 | 10 | 4.2 | | 48.1 | 5.3 | 33 | 33 | 28.7 |
| 9 | 10 | 4.35 | | | | | | |
| 10 | 10 | 3.4 | | | | | | |
| 11 | 10 | 4.0 | 129 | 38.0 | 6.7 | 41 | 36 | 16.3 |
| 12 | 10 | 3.8 | | | | | | |
| 13 | 10 | 4.6 | | | | | | |
| 14 | 10 | 3.6 | | | | | | |
| 15 | 10 | 3.0 | | | | | | |
| 16 | 5 | 3.5 | | | | | | |
| 17 | 5 | 4.2 | 113 | 37.34 | 6.4 | 36 | 57 | 0.6 |
| 18 | 5 | 3.5 | | | | | | |
| 19 | 5 | 3.1 | | | | | | |
| 20 | 5 | 3.6 | | | | | | |
| 21 | 5 | 2.8 | | | | | | |
| 22 | 5 | 3.4 | | | | | | |
| November 17, 1922 (following morning) | | | | | | | | |
| 23 | 10 | 11.2 | 111 | 52.7 | 8.8 | 34 | 37 | 20.2 |
| 24 | 10 | 5.9 | | | | | | |
| 25 | 5 | 4.2 | 93 | 51.5 | 9.1 | 34 | 35 | 21.9 |
| 26 | 5 | 4.5 | | | | | | |
| 27 | 5 | 5.2 | | | | | | |
| 28 | 5 | 3.3 | | | | | | |
| 29 | 5 | 4.1 | 109 | 52.2 | 8.0 | 33 | 31 | 28.0 |
| 30 | 5 | 3.1 | | | | | | |
| 31 | 5 | 3.0 | | | | | | |
| 32 | 5 | 2.8 | | | | | | |

*Comment.* A moderate, but rather prolonged lymphagogue effect followed the injection of tuberculin (1 cc. of 1:10,000). Lymph protein diminished but returned to the original level by the following morning. Fibrin increased in amount two to three hours after the injection and the globulins showed considerable modification. After a primary reduction in the sugar concentration, the original level was maintained throughout. In general the effect might be interpreted as one in which the endothelium

PROTOCOL 11

*Dog 472; January 24, 1923; weight 8000; inoculated December 24, 1923; tuberculin 2 cc., 1:10 O.T.*

| NUMBER | TIME | VOLUME PER TEN MINUTES | PROTEIN PER CUBIC CENTIMETER | FIBRIN | GLOBULINS | ALBUMIN | SUGAR | PHOSPHATE | $CO_2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | *mgm.* | *per cent* | *per cent* | | | | |
| 1 | 10:00–10:10 | 3.9 | 26.4 | 6.8 | 18.3 | 74.9 | 118 | 3.44 | |
| 2 | 10:10–10:20 | 4.1 | 25.1 | 4.3 | 19.8 | 75.9 | 119 | 3.33 | 0.60 |
| 3 | 10:20–10:30 | 3.2 | 25.3 | 4.0 | 16.8 | 79.2 | 115 | 3.38 | 0.63 |
| | 2 cc. 1:10 O. T. intravenously | | | | | | | | |
| 4 | 10:30–10:40 | 3.0 | 23.5 | 0 | 16.6 | 83.4 | 115 | 3.65 | 0.62 |
| 5 | 10:40–10:50 | 2.9 | 24:66 | 6.5 | 16.2 | 77.3 | 131 | 3.59 | 0.62 |
| 6 | 10:50–11:00 | 3.0 | 24.27 | 6.0 | 17.0 | 77.0 | 139 | 4.42 | 0.58 |
| 7 | 11:00–11:30 | 1.66 | 24.55 | 7.1 | 17.0 | 75.9 | 165 | 3.57 | 0.56 |
| 8 | 11:30–12:00 | 1.26 | 26.0 | 6.1 | 13.4 | 80.5 | 165 | 3.65 | 0.54 |
| 9 | 12:00–12:30 | 1.36 | 24.67 | 4.7 | 16.2 | 79.1 | 165 | 3.83 | 0.53 |
| 10 | 12:30– 1:00 | 1.43 | 25.8 | 9.0 | 19.3 | 70.7 | 154 | 4.16 | 0.55 |
| 11 | 1:00– 2:00 | 1.15 | 26.8 | 8.2 | 18.0 | 73.8 | 145 | 4.38 | 0.50 |
| 12 | 2:00– 3:00 | 1.33 | 28.8 | 9.3 | 17.3 | 73.4 | 164 | 4.16 | 0.48 |
| 13 | 3:00– 4:00 | | | | | | | | 0.46 |

had become more permeable for water and the smaller molecular aggregates. At autopsy a marked miliary tuberculosis was found in the liver, spleen and lungs.

*Comment.* A very small dose (1 cc. 1:100,000 O. T.) followed in this case by practically no lymphagogue effect but a very decided reduction in the amount of lymph protein. This is altered in the morning samples, however, when the original concentration was exceeded. Two other alterations are of interest: the increase in fibrin and the great concentration of the globulins. These latter in one pooled sample (17 to 22 make up practically

all the protein of the lymph (excluding fibrin)). Autopsy findings similar to those of dog 425.

In the following experiment the inoculation had been made with a bovine strain of relatively little pathogenicity and the animals of the series presented only insignificant healed lesions in the lungs, liver and kidney. The results of the injection of a fairly large dose of old tuberculin (2 cc. 1:10) are however of interest.

In this case the first sample of lymph collected after the injection failed to clot (a rather unusual effect) and there appears to have been no coincident increase in the globulins. No lymphagogue effect occurred, the lymph proteins diminished until toward the end of the experiment, when they increased. Fibrin increased during the same time. It will be observed that a continued increase took place in the phosphates, the sugar and the carbon dioxide diminished. The experiment indicated that the initial effect was relatively negligible but that profound changes took place at a considerable period after the injection of tuberculin.

## DISCUSSION

When we survey the alterations that are found in the thoracic lymph after the injection of tuberculin we find the results, at first glance, by no means as clear cut as those that we have dealt with in the preceding papers, but when we consider the many possibilities offered by the pathological tissue in modifying the biological reactions with which we are dealing, we believe that these experiments afford some insight into the processes. We would by no means presume to define the causes of these numerous alterations in a dogmatic manner: we can at best but surmise the possible mechanism that underlies some of them, and present the following explanation merely as a working hypothesis.

In the normal dog large doses of tuberculin bring about important alterations which involve not only the endothelium, but rapidly influence other tissues as well. As evidence of the primary stimulation we find a marked increase in phosphates, a moderate increase in lymph flow and an increase in the larger molecular

aggregates at the expense of the smaller protein fragments (protocol 7). Here, as with peptone, a second period of stimulation follows at a later period, and one that persists for a considerable length of time. But while this is taking place in the phosphates and total protein, the volume of the lymph and the sugar level are diminishing. It would seem that both an increase and a decrease in cell permeability were taking place simultaneously in different cell groups or organs.

In a general way this holds true for smaller doses of tuberculin in normal animals (protocols 2, 3 and 4). It seems probable that we still deal with a transient stimulation of the endothelium with the smaller doses, followed by a relatively prolonged impermeability (because hemoglobin injected during this time is delayed in its passage to the lymph stream) (protocols 3 and 4). But while this change occurs in the endothelium it seems probable that the liver has been stimulated and we see a late and relatively long continued increase in phosphates and sugar (protocol 3). The disturbance (perhaps associated with some degree of passive hyperemia) here is indicated furthermore by the appearance of bile pigment in the lymph. This effect of the tuberculin on the liver (and other parenchymal cells) is to be expected because the tuberculin represents a relatively small molecular aggregate which must pass rapidly from the blood to the tissues.

It is obvious that we cannot consider tuberculin as an indifferent substance for the normal organism, as has so frequently been stated in the literature of tuberculosis. While it is true that we cannot rule out the possibility that these dogs have at some time been in contact with tubercle bacilli, we have in all cases made a careful postmortem examination and have failed to find any evidence of active or healed tuberculous lesions in them.

If we now turn to the condition in the tuberculous animal we are confronted with additional complications. We deal first of all with an endothelium which is sensitized, i.e., it reacts with greater intensity and speed. The direction of that reaction is, however, the same as in the normal animal-stimulation-fatigue-death, all associated with increased permeability: the difference

lies in the relative response. If, as we have shown in the anaphylactic animal, our dose is minimal, we find only a transient increase in permeability followed again by an impermeability (reversal). In such animals we find little enzyme mobilization or increase in antithrombin, indeed we find a marked increase in the amount of fibrin coming from the liver. If our dose is larger we rapidly pass to the fatigue stage and death, with evidences of marked increase in the permeability (protein, sugar, phosphate, bile pigment, etc.). We may anticipate similar endothelial and parenchymal alterations in the tuberculous animal with tuberculin injections. Here, too, minimal doses will produce transient stimulation followed by a relatively prolonged impermeability. We must recognize that the degree of sensitization may vary, depending to some extent on the dose of the antigen, on the duration of the period during which antigenic material has come into contact with the endothelium and parenchymal cells and on the degree of intoxication existing. The importance of these alterations in endothelial sensitization becomes apparent in the tuberculin reactions that we induce in the skin or mucous membranes. In these modifications in vascular response (endothelial) are the predominating feature.

Second, we deal with tuberculous lesions scattered throughout the organism, some of which may be fixed and relatively impermeable, others of which may be recent, consisting of relatively loose aggregates of newly formed cells, with bacteria rich and toxic masses of necrotic debris at the center, the whole structure relatively permeable and particularly susceptible to alterations in permeability because at least four factors—the cells are all young, many of the cells are of endothelial origin, they are all more or less under a constant stimulus (irritation) from the toxic material of the necrotic focus, and they are specifically sensitized.

When now we inject tuberculin into a tuberculous animal we have not only to deal with the alteration in the vascular endothelium and with possible stimulation or fatigue of parenchymal cells, we also effect changes in the permeability of the tubercle. These latter changes may be initiated immediately or they may take place at a considerable time after the injection. The

changes here may be (1) a transient stimulation (with only a slight liberation of toxic material from the central necrotic focus) followed by a period of relative impermeability. (2) There may result fatigue with increased and prolonged permeability—the liberation of much toxic material from the focus—this again having a secondary effect on the vascular endothelium all over the body. This process may still be reversible and the organism recover. (3) The alteration in permeability at the focus may be so great that sufficient toxic material will be liberated to kill the animal very promptly after the injection.

We may now turn to see whether the actual findings in the lymph will fit into such a picture. First protocol 5: In this animal we deal with a natural infection with tuberculosis. A relatively large dose of tuberculin, together with hemoglobin, had been injected in the belief that we were dealing with a normal dog. Immediate evidence of an alteration of the endothelium are found. (1) The hemoglobin comes through in large concentration immediately. (2) Red blood corpuscles come through within five minutes after the injection. (3) A lymphagogue effect with (4) an increase in the protein, and (5) phosphates is noted. A little later (6) protease and (7) sugar and (8) non-protein nitrogen increase. There is little alteration in the $CO_2$ content. The larger molecular aggregates (fibrin and euglobulin) increase but inasmuch as the lymph was oxalated, we did not determine whether or not the lymph became incoagulable. When we compare this picture with the results of the injection of a similar dose of tuberculin in a normal dog the accentuation of the picture is quite striking. And if we contrast the effect with the results in dogs which have a miliary tuberculosis, the difference lies in the fact here we find no evidences of a second intoxication, at least in the time during which the animal was under observation. Volume, protein, protease, non-protein nitrogen all gradually diminish, although phosphates remain higher than before the injection and relatively we have more fibrin and globulins. There is no reason to suspect that additional toxic material has originated in the tuberculous foci. This corresponds with the autopsy findings, which are those of a typical

healed tuberculosis with small gelatinous fibrous lesions in the lungs, minute, non-necrotic lesions in the liver and a firm fibrous scar in the kidney.

When now we turn to animals that have a *miliary tuberculosis* with large amounts of *necrotic material scattered* throughout the body we find a different picture. In protocol 6 the injection of a relatively large dose (0.5 cc.) is followed by a transient lymphagogue effect with an increase in protein (endothelial stimulation) then a period with a reduction in protein to be followed by a prolonged increase in permeability indicated by the increase of the protein to 52 mgm. per cubic centimeter. This secondary effect would correspond to the effect of a liberation of toxic material from the foci of infection.

In the second protocol of this series (protocol 7) we deal with an animal which already has a permeable endothelium, the amount of protein contained in the lymph being almost as great as that of the normal blood plasma. Blood corpuscles enter the lymph stream half an hour after the injection of tuberculin.

With a smaller dose, however, the injury from the tuberculin is less and we find only a diminution in the amount of protein in the lymph after the injection of the tuberculin. In protocol 8 the animal was very restless and the experiment was therefore terminated about three hours after the injection. Whether a secondary rise would have occurred here is problematical.

With still smaller doses (1:10,000) no increase in protein of the lymph takes place even when examined the following day although some stimulation of the liver seems apparent in the increase in fibrin two to three hours after the injection. Here, then, the dose has been sufficiently small so that no increase in permeability of endothelium (except a transient increase in the rate of flow) becomes apparent, and evidently no increase in permeability of the numerous tuberculous foci in the liver, lungs, and spleen has occurred.

Another animal of the same series, however, seems more sensitive (protocol 10). In this animal the injection of tuberculin 1:100,000 was followed by a reduction in the amount of protein in the lymph for the first two hours, but the following morning

the amount had greatly increased and hemoglobin injected during this time appeared in the lymph five minutes after the injection.

These experiments would indicate that the effect of tuberculin in the tuberculous dog, is, as we have suggested, twofold—very small doses may be followed by a transient stimulation of the endothelium, followed by a recovery during which time the endothelium is relatively less permeable. This is in some animals followed by an alteration of the permeability of the focus, resulting in an increased liberation of toxic material. When the lymph of such animals is examined the following day, the concentration of the proteins is increased, i.e., the endothelium has become relatively fatigued.

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# THE FAILURE OF PEPTONE TO PROTECT AGAINST ANAPHYLACTIC SHOCK AND ALLERGIC CONDITIONS[1]

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During the past few years, there has been considerable interest shown in the use of peptone in allergic conditions. Pagniez and Vallery-Radot (1) and Widal, Abrami, and Brissaud (2) maintain that in patients with urticaria, peptone, taken by mouth before the ingestion of the offending protein, will prevent the eruption as well as its antecedent hemoclasic crisis. Auld (3, 4) has had considerable success in the treatment of bronchial asthma by intravenous and intramuscular injections with a mixture of Witte's and Armour's peptone suspended in 0.85 per cent NaCl solution. He attributes this to a colloidal readjustment within the body and consequent prevention of colloidoclasis.

In 1909, Biedl and Kraus (5) experimenting with dogs sensitized to horse serum, claimed that a sublethal dose of Witte's peptone protected the sensitized animal against the shocking dose of horse serum. They maintained that there was a physiological identity between the action of Witte's peptone and anaphylactic poisoning. These observations were partially corroborated by Arthus (6, 7). On the other hand, Manwaring (8) was unable to confirm the identity of the action of peptone with anaphylaxis. Novy, De Kruif, and Novy (9) stated that in guinea-pigs and in rabbits, peptone injected intravenously caused a death similar to that of anaphylactic shock.

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Spolverini (10), in order to prevent anaphylactic symptoms, gave peptone to children who had received diphtheria antitoxin. He claimed complete success. Auld (4) quoted Dale as having remarked that peptone does protect the sensitized guinea-pig from shocking doses of the injected protein.

## EXPERIMENTAL

The following experiments were done to determine whether peptone under given conditions would protect sensitized guinea-pigs against anaphylactic shock.

In the first set of experiments, egg white was the antigen used. This was prepared by mixing equal parts by volume of the white of freshly laid hens' eggs and 0.85 per cent NaCl solution. The antigen was freshly prepared for each series of injections.

The peptone employed was a 5 per cent suspension in 0.85 per cent NaCl solution, of a mixture of Armour's and Witte's peptones, in ratio of 3:1. It was prepared according to the method described by Auld, as follows: The peptone was dissolved in the hot saline solution (56°C.) and then made faintly alkaline to litmus with N/1 sodium carbonate solution. It was next placed in the water-bath at 56°C. for thirty minutes and filtered while hot. Enough phenol was added to make a final concentration of 0.5 per cent. Its sterility was then tested.

All the injections in the following experiments were given intracardially and twelve days were allowed to elapse between the sensitizing and shocking doses.

*Experiment 1* (control experiment). To determine the effectiveness of egg white suspension as an anaphylactogen. The sensitizing dose was 1 cc. of the prepared egg white suspensions given intracardially. The shocking dose was 0.2 c.c. of the same suspension given intracardially and the interval between the two was twelve days.

In the following series of experiments, the various degrees of shock are indicated by the following terms: slight shock (hair ruffled; slight dyspnea, restlessness, and scratching of nose); moderate shock (hair ruffled, coughing, moderate dyspnea, slight

paralysis of hind legs); severe shock (hair ruffled, scratching of nose, coughing, acute dyspnea, convulsions, recovery rapid).

These experiments show that egg white as used here is not a very potent agent for inducing anaphylactic shock. This is possibly due to the short time interval allowed between the sensitizing and shocking doses. It would seem that with such a mild antigen any effect that peptone might have in producing antianaphylaxis, should be apparent.

TABLE 1

*Control experiment without peptone*

| PIG NUMBER | WEIGHT | INITIAL DOSE OF EGG WHITE (1:1) | SYMPTOMS | SHOCKING DOSE OF EGG WHITE (1:1) | RESULT |
|---|---|---|---|---|---|
| | *grams* | *cc.* | | *cc.* | |
| 1 | 300 | 1 | None | 0.2 | Died in 2.5 minutes |
| 2 | 244 | 1 | None | 0.2 | Slight shock |
| 3 | 234 | 1 | None | 0.2 | Died in 4 minutes |
| 4 | 385 | 1 | None | 0.2 | Slight shock |
| 5 | 421 | 1 | None | 0.2 | No shock |
| 6 | 400 | 1 | None | 0.2 | No shock |
| 7 | 308 | 1 | None | 0.2 | Died in 3 minutes |
| 8 | 422 | 1 | None | 0.2 | Slight shock |
| 9 | 361 | 1 | None | 0.2 | Moderate shock |
| 10 | 371 | 1 | None | 0.2 | Slight shock |
| 11 | 445 | 1 | None | 0.2 | Died in 3.5 minutes |
| 12 | 401 | 1 | None | 0.2 | Died in 5.5 minutes |
| 13 | 420 | 1 | None | 0.2 | Died in 2.5 minutes |
| 14 | 460 | 1 | None | 0.2 | Slight shock |
| 15 | 375 | 1 | None | 0.2 | Slight shock |
| 16 | 365 | 1 | None | 0.2 | Slight shock |

Totals: Died, 6; moderate shock, 1; slight shock, 7; no shock, 2.

*Experiment 2.* To determine whether peptone given intracardially thirty minutes before the shocking dose will prevent anaphylactic symptoms.

One cubic centimeter of egg white suspension was injected intracardially into each of 25 guinea-pigs. Twelve days later, the 5 per cent peptone suspension was similarly injected in amounts varying from 0.5 to 2 cc. as indicated in the table. This was followed thirty minutes later by 0.2 cc. of egg white suspension.

The percentage of deaths in this experiment was higher than in the control experiment, showing that peptone as here used gave no protection.

TABLE 2

| PIG NUMBER | WEIGHT | INITIAL DOSE OF EGG WHITE (1:1) | SYMPTOMS | PREVENTIVE DOSE OF PEPTONE* | RESULT OF SHOCKING DOSE OF EGG WHITE (0.2 cc.) |
|---|---|---|---|---|---|
| | *grams* | *cc.* | | *cc.* | |
| 1 | 245 | 1 | None | 0.5 | Died in 4 minutes |
| 2 | 294 | 1 | None | 0.5 | Died in 4 minutes |
| 3 | 327 | 1 | None | 0.5 | Died in 3 minutes |
| 4 | 292 | 1 | None | 0.8 | Slight shock |
| 5 | 299 | 1 | None | 0.8 | Died in 3 minutes |
| 6 | 214 | 1 | None | 1.0 | No shock from egg, died later from effect of peptone |
| 7 | 179 | 1 | None | 1.0 | No shock |
| 8 | 300 | 1 | None | 1.0 | Slight shock |
| 9 | 442 | 1 | None | 1.0 | Slight shock |
| 10 | 238 | 1 | None | 1.0 | Slight shock |
| 11 | 359 | 1 | None | 1.0 | Slight shock |
| 12 | 234 | 1 | None | 1.0 | Moderate shock |
| 13 | 277 | 1 | None | 1.0 | Severe shock |
| 14 | 223 | 1 | None | 1.0 | Died in 2.5 minutes |
| 15 | 247 | 1 | None | 1.0 | Died in 4.5 minutes |
| 16 | 226 | 1 | None | 1.0 | Died in 2.5 minutes |
| 17 | 239 | 1 | None | 1.0 | Died in 3.5 minutes |
| 18 | 254 | 1 | None | 1.0 | Died in 4 minutes |
| 19 | 287 | 1 | None | 1.0 | Died in 3.5 minutes |
| 20 | 397 | 1 | None | 1.0 | Died in 4.5 minutes |
| 21 | 330 | 1 | None | 1.5 | Died in 3.5 minutes |
| 22 | 270 | 1 | None | 1.5 | Slight shock |
| 23 | 254 | 1 | None | 1.5 | Moderate shock |
| 24 | 233 | 1 | None | 1.5 | Severe shock |
| 25 | 304 | 1 | None | 2.0 | Died in 3.5 minutes |

Totals: Died, 13; severe shock, 2; moderate shock, 2; slight shock, 6; no shock, 2.

* Peptone thirty minutes before 0.2 cc. of egg white.

In each case of anaphylactic death, an autopsy was performed. Each presented practically the same picture: lungs tremendously distended and pinkish white in color, sometimes with petechial hemorrhages. In contrast to this, the guinea-pig in this experi-

ment which died from an injection of peptone showed normal lungs and marked congestion of the intestines.

*Experiment 3.* To determine whether peptone injected at intervals following the initial dose of egg white protects the guinea-pig from symptoms following the shocking dose.

One cubic centimeter of egg white suspension was injected intracardially into each of 12 guinea-pigs. They then received

TABLE 3

| PIG NUMBER | WEIGHT | INITIAL DOSE OF EGG WHITE (1:1) | SYMPTOMS | PEPTONE IN SERIES (5 DOSES) | SYMPTOMS | FINAL DOSE OF PEPTONE† | RESULTS OF SHOCKING DOSE OF EGG WHITE (0.2 cc.) |
|---|---|---|---|---|---|---|---|
| | *grams* | *cc.* | | *cc.* | | *cc.* | |
| 1 | 242 | 1 | None | 0.5 | None | — | Died in 4.5 minutes |
| 2 | 230 | 1 | None | 0.5 | None | — | Died in 4 minutes |
| 3 | 231 | 1 | None | 0.5 | None | 1.0 | No shock |
| 4 | 210 | 1 | None | 0.5 | None | 1.0 | Died |
| 5 | 229 | 1 | None | 0.5 | None | 0.5 | Slight shock |
| 6 | 320 | 1 | None | 0.5 | None | 1.0 | No shock |
| 7 | 275 | 1 | None | 1.0 | None | 1.0 | No shock |
| 8 | 310 | 1 | None | 1.0 | Slight tremor at first injection | 1.0 | Died in 4.5 minutes |
| 9 | 317 | 1 | None | 1.0 | Slight tremor at first injection | 1.0 | Slight shock |
| 10 | 262 | 1 | None | 1.0 | Slight tremor at first injection | 1.0 | Moderate shock |
| 11 | 264 | 1 | None | 1.0 | Slight tremor at first injection | 1.0 | Slight shock |
| 12 | 350 | 1 | None | 1.0 | Slight tremor at first injection | 1.0 | Died in 3.5 minutes |

Totals: Died, 5; moderate shock, 1; slight shock, 3; no shock, 3.

† Peptone thirty minutes before egg white.

at intervals of forty-eight hours an injection of the peptone suspension, varying in amount from 0.5 to 1 cc. as indicated in table 3. Also, in some cases, 1 cc. of peptone was injected thirty minutes before the shocking dose. The shocking dose was 0.2 cc. of egg white suspension given intracardially, twelve days after the sensitizing dose.

The percentage of deaths in this experiment is essentially the same as in the control experiments, showing that peptone as here used gives no protection.

To the 13 guinea-pigs that had survived in all the experiments with slight or no symptoms, a single injection of peptone was given at intervals of time varying from two to sixteen days later than the date of the shocking dose of egg white. These injections were followed thirty minutes later by injections of egg white suspension and death or severe symptoms occurred in every instance. Rather than attribute this to any sensitizing property of peptone, it is inferred that shock occurred more uniformly because a longer period of time had elapsed between the sensitizing and shocking doses than had occurred in experiments 1, 2, and 3.

In order to confirm the foregoing results by a more accurate method, the Dale technique (11, 12) was used to record the following experiments.

According to him, the uterus from a sensitized guinea-pig when suspended in a bath of Locke's or Ringer's solution, will respond to minute quantities of the protein to which it has been sensitized, when this is added to the bath. An accurate measurement of the contractions is recorded on smoked paper by means of a kymograph and a recording lever. The best results are obtained with virgin guinea-pigs averaging not more than 250 grams in weight. The following is the formula of the Locke's solution used: NaCl 9 grams, KCl 0.42 gram, $CaCl_2$ 0.24 gram, $NaHCO_3$ 0.5 gram, dextrose 1 gram, distilled water 1000 cc.

This method has also been used, in determining anaphylactic response, by Weil (13), and many other investigators.

A similar technic has also been used to show the effect of peptone on the smooth muscles of various animals. It was used by Dale and Laidlaw (14) who described the stimulating effect of Witte's peptone on the uterus of the guinea-pig. Roger (15) reported the same effect on the intestine of the rabbit.

Olivecrona (16), in an interesting series of experiments on cats' and rabbits' intestines, showed that while Fairchild's peptone and histamin in weak concentrations stimulated the muscles, in strong concentrations both Fairchild's peptone and histamin paralyzed the smooth muscles of the intestine for several hours. This peptone in concentrations of 1:200 and 1:250 pro-

duced paralysis of the cat's intestinal muscle. Abel and Macht (17) found a similar difference in the action of weak and strong concentrations of histamin on the guinea-pig uterus. Increase of concentration beyond a definite limit caused relaxation and inhibition of the muscular contractions, instead of the stimulation produced by weak concentrations of the same substance. In the experiments described below, concentrations of mixed peptone from 1:396 to 1:520 caused complete inhibition of the guinea-pig uterus. Concentrations as weak as 1:10,000, while causing no perceptible stimulation, were followed by smaller curves of response to the specific protein than the control curves from strips to which peptone had not been added.

*Technic.* The uteri of the first 17 guinea-pigs that were tested by the Dale technic were cut in segments so that sometimes as many as four different experiments were performed on 1 guinea-pig. Thus the last segment of uterus tested had usually been standing for several hours. As it naturally did not respond as well as when the uterus had been freshly excised, it wäs difficult to control the experiment properly. So another machine, in which two segments of the uterus, one previously treated with peptone and the other not so treated, could be tested simultaneously, was devised in order to furnish an accurate control. To control the peptone a 0.85 per cent NaCl solution was used, and a period of about four minutes was allowed to elapse before adding the egg white suspension.

Forty-one guinea-pigs were sensitized by intraperitioneal injection with 1 cc. of egg white suspension (1:1) in 0.85 per cent NaCl solution. The Dale experiment was performed three to five weeks later. The smallest amount of peptone used in the bath was 4 cc. of a 5 per cent suspension. In this concentration (1:1270) no protection was noted (chart 1); with 6 cc. and 8 cc. of peptone, response to the protein was diminished (charts 2 and 3), whereas in amounts of 10 cc. and over (concentration of 1:508 or stronger), inhibition of the response was complete (charts 4, 5, 6a and 6b).

The inhibition noted above is paralleled by the control experiment, in which 12 cc. of the peptone suspension produced inhibi-

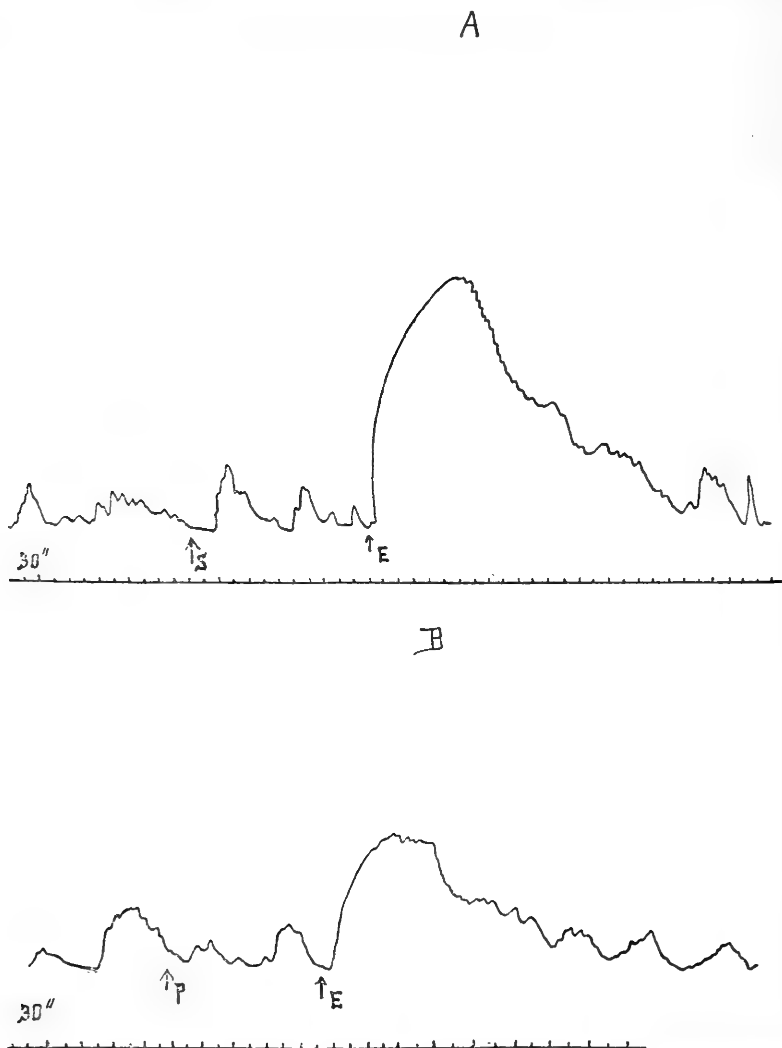
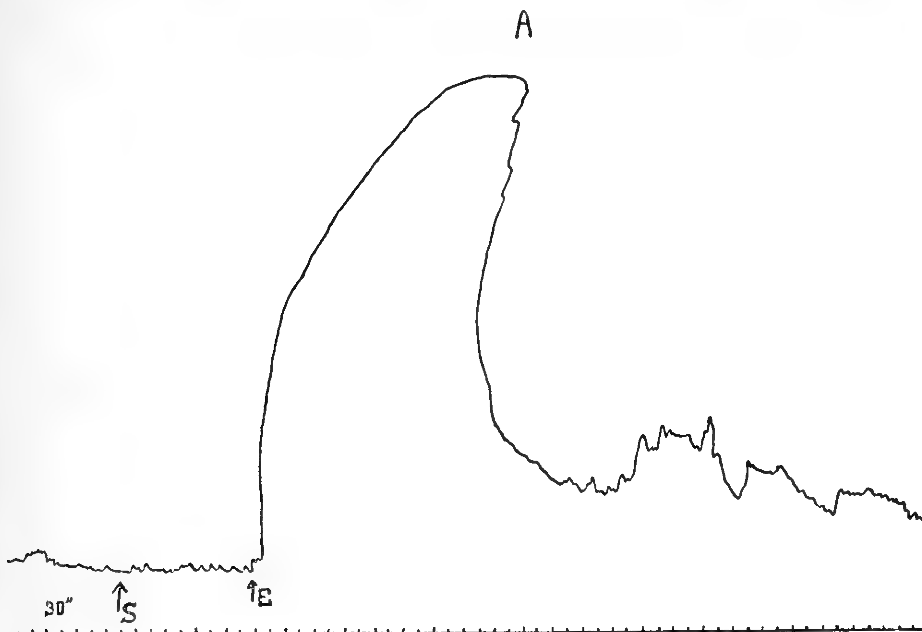



CHART 1. EFFECT OF PEPTONE (1:1270) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA PIG

*Simultaneous records*

A Control segment {S: 4.0 cc. NaCl 0.85 per cent; E: 0.3 cc. egg white (1:1)}

B. Reaction segment {P: 4.0 cc. peptone A. & W. 5 per cent; E: 0.3 cc. egg white (1:1)}



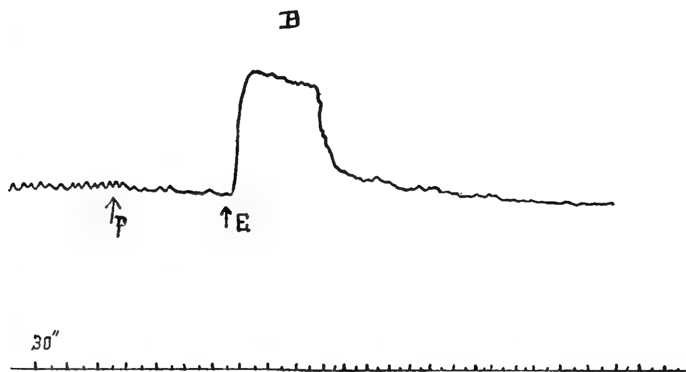




CHART 2. EFFECT OF PEPTONE (1:846) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA PIG

*Simultaneous records*

A. Control segment { S: 6.0 cc. NaCl 0.85 per cent
E: 0.3 cc. egg white (1:1)

B. Reaction segment { P: 6.0 cc. peptone A. & W. 5 per cent
E: 0.3 cc. egg white (1:1)

tion of rhythmic contractions and loss of tone in the normal unsensitized guinea-pig uterus under the conditions of the Dale experiment (chart 7). This action of peptone is evidently similar to the effect of histamine and peptone on the normal smooth muscles of other animals when in strong concentration (Olivecrona, Abel and Macht).

*Clinical.* At the Protein Sensitization Department of the Cornell Clinic, the same peptone suspension which was used experimentally was given intravenously according to Auld's method in

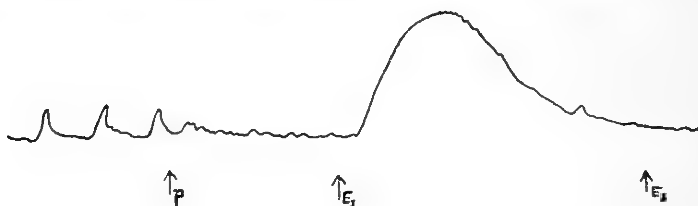


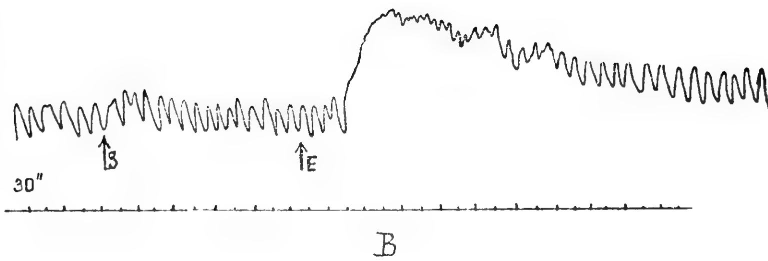

CHART 3. EFFECT OF PEPTONE (1:635) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA-PIG

P: 8.0 cc. peptone A. & W. 5 per cent
$E_1$: 0.3 cc. egg white (1:1)
$E_2$: 0.5 cc. egg white (1:1)

only 9 cases of bronchial asthma. Other measures had been tried without distinct success. The peptone suspension was given at intervals of three to four days, allowing thereby two injections a week. The initial dose was 0.1 cc. given intravenously and at each successive injection, this dose was raised 0.2 cc. until 1.3 cc. was reached. This dose was repeated six times.

In 2 instances, there were severe symptoms following the initial injections. In one, this was characterized by a sudden onset of dyspnea of asthmatic type, cyanosis, and syncope, immediately following the injection of peptone. After thirty minims of epinephrin, solution (1:1000), in two equal doses had been injected

A




B

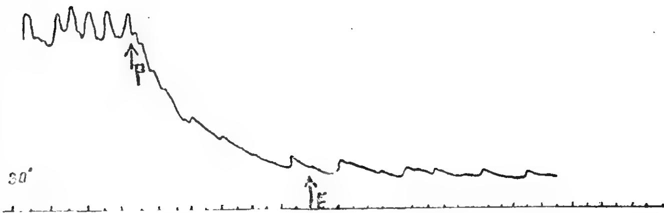



CHART 4. EFFECT OF PEPTONE (1:508) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA-PIG

*Simultaneous records*

A. Control segment { S: 10.0 cc. NaCl 0.85 per cent
E: 0.3 cc. egg white (1:1)

B. Reaction segment { P: 10.0 cc. peptone A. & W. 5 per cent
E: 0.3 cc. egg white (1:1)

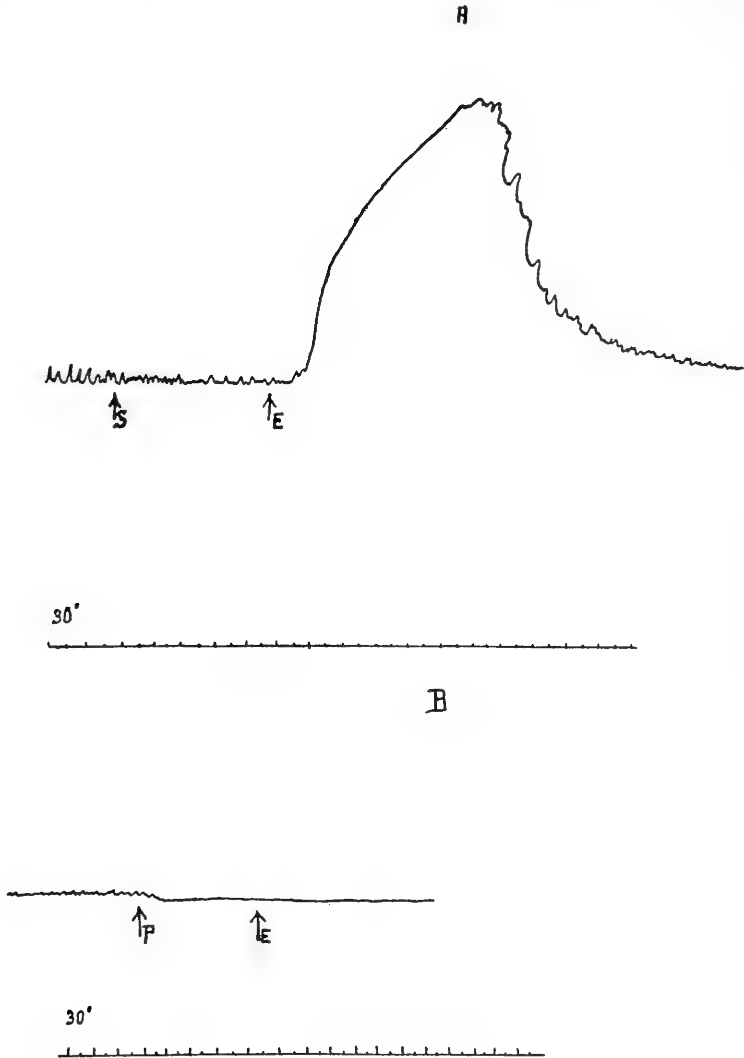



CHART 5. EFFECT OF PEPTONE (1:460) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA-PIG

*Simultaneous records*

A. Control segment { S: 11.0 cc. NaCl 0.85 per cent; E: 0.5 cc. egg white (1:1) }

B. Reaction segment { P: 11.0 cc. peptone A. & W. 5 per cent; E: 0.5 cc. egg white (1:1) }

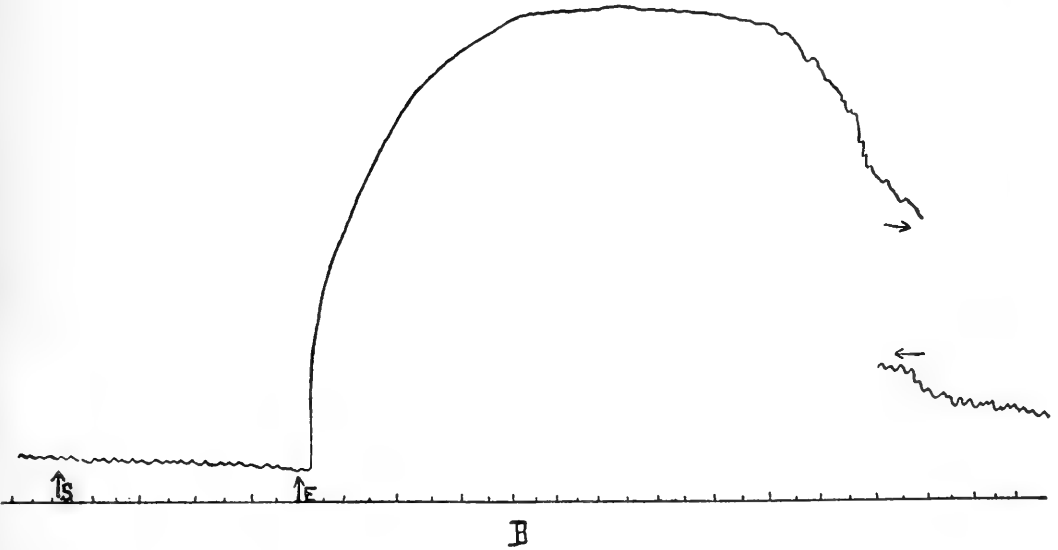
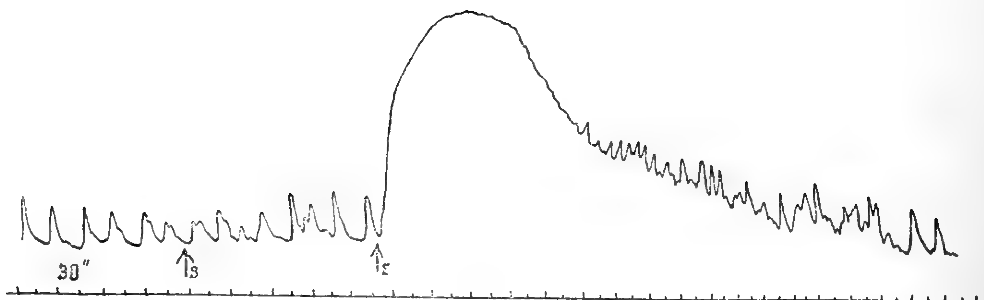


CHART 6a. EFFECT OF PEPTONE (1:424) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA-PIG

*Simultaneous records*

A. Control segment { S: 12.0 cc. NaCl 0.85 per cent; E: 0.3 cc. egg white (1:1) }

B. Reaction segment { P: 12.0 cc. peptone A. & W. 5 per cent; E: 0.3 cc. egg white (1:1) }



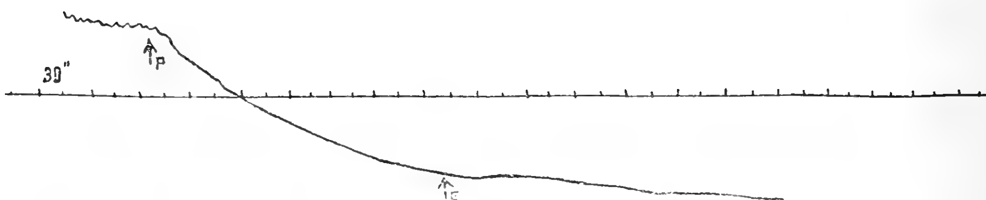




CHART 6b. EFFECT OF PEPTONE (1:424) ON SENSITIZED UTERINE MUSCLE STRIP OF GUINEA-PIG

*Simultaneous records*

A. Control segment { S: 12.0 cc. NaCl 0.85 per cent; E: 0.4 cc. egg white (1:1)

B. Reaction segment { P: 12.0 cc. peptone A. & W. 5 per cent; E: 0.4 cc. egg white (1:1)

subcutaneously, recovery was rapid and apparently complete within an hour. It was subsequently found that this patient gave a positive intradermal reaction to the peptone suspension used. Peptone given by mouth thereafter caused no untoward reaction and was apparently very effective in preventing further asthmatic paroxysms. Various other measures had been used previously. Another patient developed acute symptoms—diarrhea, vomiting, and severe abdominal cramps following the injection and lasting several days. Excepting the first case mentioned and one other case, in which the patient was apparently relieved of asthmatic paroxysms following the course of peptone injec-

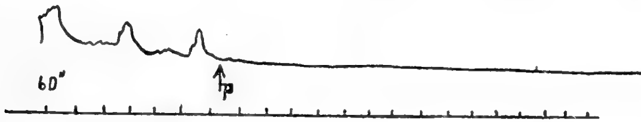



Chart 7. Effect of Peptone (1:424) on Uterine Muscle Strip of Normal Guinea-Pig
P: 12.0 cc. peptone A. & W. 5 per cent

tions, the results obtained were not impressive. Occasionally, there was a temporary freedom from attacks, but only for a short time, even while treatment was constant.

A few cases of bronchial asthma were treated by mouth, with Chapoteaut's Peptone tablets.[2] The results were negative in this safer method of administration. Patients with urticaria were given peptone tablets also in doses from 0.1 to 0.3 gram fifteen minutes before meals. The results were wholly disappointing.

## SUMMARY

1. Guinea-pigs sensitized to egg white, receiving peptone intracardially thirty minutes before the shocking dose of egg white, showed no protection against anaphylactic shock in comparison with sensitized guinea-pigs not treated with peptone.

2. Guinea-pigs sensitized to egg white and receiving thereafter serial intracardial peptone injections were similarly found to be unprotected against anaphylactic shock.

[2] A commercial preparation of peptone obtained from E. Fougera, 90 Beekman Street, New York City.

3. Peptone in weak concentration did not prevent the specific reaction of the uterine muscle strip to egg white in the Dale experiment.

4. Peptone in strong concentrations produced inhibition of the of the specific reaction of the uterine muscle strip to egg white in the Dale experiment. Inhibition of rhythmic contractions and loss of tone were also observed in the normal unsensitized muscle under the conditions of the Dale experiment when the concentration of peptone was sufficiently strong.

5. Clinically, the use of peptone intravenously in bronchial asthma according to the method of Auld has been disappointing and is considered dangerous.

The authors express their appreciation to Miss M. M. Owings for her technical assistance.

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# A DANGEROUS "UNIVERSAL DONOR" DETECTED BY THE DIRECT MATCHING OF BLOODS

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The ideal donor for the transfusion of blood has long been known to be one of the same group as the patient. It is not always possible for these conditions to obtain, and a person of group I (Jansky) has been considered as a safe donor for any of the groups. This may not always be the case, as will be shown in this communication.

In determining blood compatibilities, two methods have been employed, the indirect and the direct. The indirect method groups the patient and prospective donors among the four recognized classes, and permits of the selection of a donor belonging to the same class as the patient, if such a donor is available. Otherwise, the "universal donor" must be resorted to.

For the proper performance of the indirect method, stock sera of Groups II and III, and a drop of the patient's citrated blood are the essential requirements.

The direct method is generally, but not universally, employed, just prior to transfusion, and one object of this test should be to duplicate in vitro, so far as is possible, the conditions of the transfusion. Several technics have been described, such as those of Weil, of Rous and Turner, and of Coca. The last modification is the simplest, and, in the proportion of the bloods used, it approximates closely enough the proportions usually obtaining in the transfusion. It was in doing this test, as described below, that we first noted the phenomenon here reported.

Donor and recipient are prepared as for a blood count. The necessary equipment is one white cell counting pipet, a hagedorn needle,

10 per cent sodium citrate, normal saline solution, three clean glass slides and cover slips. The pipet is rinsed with the citrate solution, and enough solution is left to fill the terminal $\frac{1}{10}$ division. The finger of the recipient is punctured, and when the drop is large enough, blood is drawn into the pipet up to the 1.0 mark. This is then blown out on the right end of slide I (fig. 1) (at *R*) and mixed with the end of the pipet.

The same procedure is then carried out for the donor's blood with the same pipet, after it has been well rinsed with the saline solution, and the drop is placed at the left end of slide I (D). Enough of the donor's

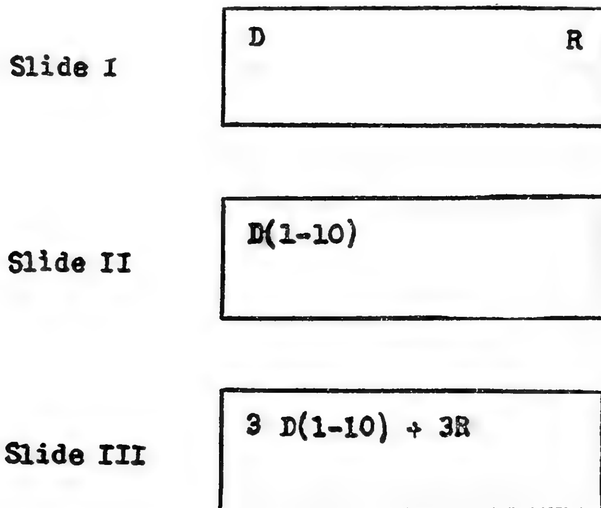


Fig. 1

citrated blood is left in the pipet to fill the terminal $\frac{1}{10}$ division, and saline solution is drawn in with the blood up to 1.0 mark. The contents are blown out on slide II, and mixed with the end of the pipet. This gives a $\frac{1}{10}$ dilution of the donor's blood, which is the dilution that ultimately results when 500 cc. of blood (the usual amount) are transfused into a patient, the total volume of whose blood is estimated at 5000 cc.

Three divisions ($\frac{3}{10}$) of the diluted donor's blood (slide II) are drawn into the pipet, and also three divisions of the recipient's citrated whole blood (*R*, slide I). The contents of the pipet are blown out on the third slide, thoroughly mixed with the end of the pipet, and covered with a cover glass.

This mixture is carefully watched for agglutination, which is hastened by moving the cover slip a bit from time to time. If the reaction is marked, it is easily seen macroscopically, but, if doubtful, the microscope must be used. Great care is necessary not to mistake rouleaux formation for agglutination. Movement of the cover slip generally breaks up rouleaux. A reaction is read as negative if no agglutination appears at the end of fifteen minutes. It is also possible to determine whether the recipient's or donor's cells are agglutinated. In the former case, the great majority of the cells (i.e., $\frac{9}{10}$) are clumped, while $\frac{1}{10}$ are free; in the latter case, the reverse of this is found.

TABLE 1

| | JANSKY | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Serum | ab | b | a | 0 |
| Cells | 0 | A | B | AB |

In a class demonstration of this method, it was found that when L.,[1] group II (Jansky), was used as a donor, and M.,[1] group I (Jansky), as recipient, agglutination of the donor's corpuscles took place. This reaction is normal, because group I serum, which contains agglutinins a and b is receiving agglutinable cells from group II. When, however, M., group I, was used as donor, and L., group II, as recipient, agglutination also resulted, affecting most of the corpuscles in the mixture. This last reaction was not expected, because the cells of group I have no agglutinable substances in them, and the serum in the test was diluted 1:10, in which dilution the agglutinating power of the group I serum is supposedly sufficiently weakened to prevent clumping of the agglutinable group II cells. It is for these two reasons that the group I individual is considered a "universal donor." The test was repeated, and the result was found to be the same.

The possibility that the unusual clumping represented an auto-agglutination of L.'s corpuscles was considered, and this explanation could be excluded by the observation that the mixture of L.'s plasma and corpuscles presented no clumping unless M.'s plasma was added. Furthermore, as will be presently shown (table 2)

[1] L.-Levine; M.-Mabee.

the degree of agglutination of L.'s washed blood cells was proportional to the quantity of added M.'s plasma or serum.

We determined the agglutinating power of M.'s serum in the following manner:

Defibrinated blood of M. and of L. was centrifugalized, and the sera were pipetted off. A 1:4 suspension of L.'s washed corpuscles was prepared by adding 1.5 cc. of normal saline to 0.5 cc. of the corpuscular sediment. One-tenth of a cubic centimeter of this suspension was put into each of five tubes. To these tubes, a 1:40 and a 1:400 dilution of M.'s serum were added, as follows: 0.5, 0.2, 0.1 and 0.05 cc. of the 1:40 dilution and 0.3 cc. of the 1:400 dilution. The contents were mixed by shaking gently, and contrifugalized for two minutes at high speed. Each of the five tubes showed agglutination in varying degrees, as evidenced by the completeness with which the cells stayed together on gentle shaking, and by failure of clouding the supernatant fluid. This demonstrated the high agglutinating power of M.'s serum for L.'s cells.

TABLE 2

| | TUBE 1 | TUBE 2 | TUBE 3 | TUBE 4 | TUBE 5 |
|---|---|---|---|---|---|
| L. cells, 1:4 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| M. serum 1:40 | 0.5 | 0.2 | 0.1 | 0.05 | |
| M. serum 1:400 | | | | | 0.3 |
| Gross agglutination in test tube | ++++ | ++ | ++ | + | ± |

The following experiment was carried out to determine (1) the specificity of the action of M.'s serum upon L.'s cells, and (2) the agglutinating power of other group I sera. With this in view, a number of individuals were grouped and bled. The defibrinated blood was centrifugalized, the serum separated, and the cells (i.e., those of not group I) were washed once with sterile saline solution. In this study the serum of group I individuals and the cells of those not in group I were used. A 50 per cent suspension of the washed cells was made by taking 0.5 cc. of the cell sediment and 0.5 cc. of sterile saline solution. Dilutions of all sera (M.'s serum included) of 1:2, 1:5, and 1:10, were prepared. Each serum in the three dilutions was tested with as

many cells as feasible. A drop of each was put on a slide, thoroughly mixed, covered with a cover slip, and watched for agglutination. M.'s serum was tested against each cell suspension. The experiment was planned to simulate conditions as they obtain in the human body; the 50 per cent cell suspension in saline representing the 50 per cent cells in the human blood; the 1:10 serum dilution representing the ratio of donor's blood in the recipient's veins. A 2:10 dilution is used for the purpose of detecting what would happen if 1000 cc. were the amount transfused instead of 500 cc. The results of these tests are shown in table 3.

TABLE 3

*Sera (Group I)*

| CELLS (NOT GROUP I) | M | | | 1–8 | | | 9–13 | | | 14–17 | | | 18 | | | 19–20 | | | 21–25 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 | 1:2 | 1:5 | 1:10 |
| a | + | + | + | + | − | − | | | | | | | | | | | | | | | |
| b | + | + | + | + | − | − | | | | | | | | | | | | | | | |
| c | + | + | − | + | − | − | | | | | | | | | | | | | | | |
| d | + | + | + | + | − | − | | | | | | | | | | | | | | | |
| e | + | + | + | + | − | − | + | − | − | | | | | | | | | | | | |
| f | + | + | + | + | − | − | + | − | − | + | − | − | + | + | − | + | − | − | | | |
| g | + | + | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | | | |
| h | + | + | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| i | + | + | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| j | + | + | + | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| k | + | + | + | | | | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| l | + | + | + | | | | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| m | + | + | + | | | | + | − | − | + | − | − | + | − | − | + | − | − | + | − | − |
| n | + | + | + | | | | | | | + | − | − | + | − | − | + | − | − | + | − | − |
| o | + | + | + | | | | | | | + | − | − | + | − | − | + | − | − | + | − | − |
| p | + | + | + | | | | | | | | | | + | − | − | + | − | − | + | − | − |
| q | + | + | + | | | | | | | | | | + | − | − | + | − | − | + | − | − |

A perusal of table 3 shows the following:

1. M.'s serum diluted 1:10 agglutinated all but one of the cells, c, which however, was agglutinated in a 2:10 dilution.

2. Of the 26 group I sera tested, M.'s serum was the only one with this remarkably strong agglutinating power. One serum, no. 18, agglutinated cells f in a 2:10, but not in a 1:10 dilution.

These findings lead us to attach far more importance and dignity to the direct test than is indicated in the literature. Meleney concludes "that where a reliable laboratory is at hand, the direct grouping of donor and recipient is *not* necessary, but should otherwise be done." Nevertheless, were M. to act as "universal donor" to any of the 17 patients (not group I) tested, with one possible exception, 250 cc. of her blood or one-half of the usual amount would cause a most complete and colossal agglutination of the recipient's cells with the formation, as Dr. Conner suggests, of multiple thrombosis and embolism and instantaneous death.

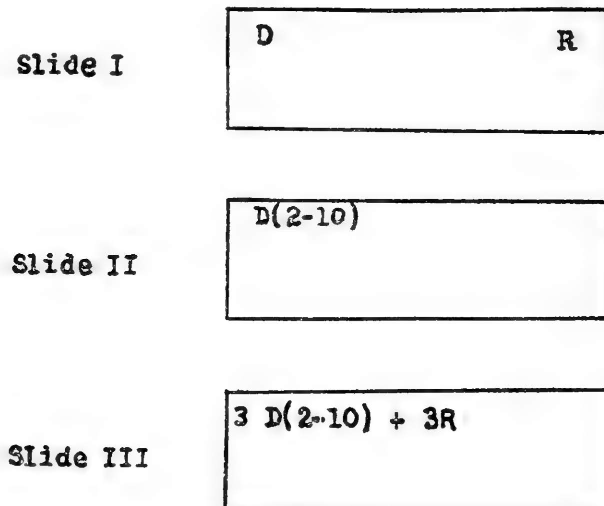


Fig. 2

On the other hand, when an emergency transfusion is needed, the procedure is justified without a previous grouping, provided the direct test shows no agglutination. In other words, it is safer to use the direct test than to depend upon a "universal donor." It is not the intention of the writers to minimize the importance of the indirect test. The best donor is one out of the same group as the recipient, and this can be determined by the use of the indirect test with stock sera of groups II and III. However, the direct test should always be performed prior to any

transfusion to check up a possible error in grouping, and, in the light of the present report, to detect an unusually high-titred serum in a group I individual who is offering himself *for the first time* as a "universal donor."

The foregoing experiences make it appear advisable to introduce a quantitative modification in the technic of the direct test of Coca.

The fact that the serum of the group I individual 18 was able, in a 2:10 dilution, to clump the f agglutinable corpuscles would seem to mark individual 18 as an undesirable donor, if not actually a dangerous one. On the other hand, it seems safe to assert that any group I individual can be accepted as a universal donor, whose serum in a dilution of 2:10 causes no microscopically detectable clumping of the recipient's corpuscles.

We recommend, therefore, that, in performing the direct matching test of Coca, the donor's citrated blood be diluted with saline solution 2:10 instead of 1:10 before being mixed with the equal volume of recipient's citrated blood (fig. 2).

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# ENVIRONMENTAL CONDITIONS INFLUENCING COMPLEMENT FIXATION

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Several years ago, while making many routine examinations of spinal fluid it was occasionally disconcerting to find one which had a normal cell count, negative globulin reaction and yet which gave a positive Wassermann test. In checking them up, it was found that the individual making the cell counts had, at times, introduced a small amount of acetic acid into the specimen of spinal fluid. The technic employed was that of drawing glacial acetic acid into a white blood pipette and then blowing the excess of acid from the pipette. The spinal fluid was then drawn into the pipette, the small amount of acid which remained in the pipette being sufficient to differentiate the white cells for counting. When a small amount of fluid was withdrawn from the main bulk and used only for the Wassermann test the difficulty cited above was no longer encountered.

With the above observations as a basis it was decided to study the effect of acetic acid on Wassermann negative spinal fluids and blood sera and, if possible, to determine the mechanism by which the result of the reaction was altered. The substance in question is one which exhibits both lytic and antilytic effects. In this respect acetic acid is not unlike a wide variety of substances which have been found to exhibit similar action. Thus, Hektoen pointed out that some of the inorganic salts exerted an inhibiting effect upon the hemolytic reaction when present in certain concentrations. Later Hektoen and Ruediger, as well as Manwaring, found that small doses of solutions of many salts prevent the lysis of red corpuscles and of bacteria by immune sera. These authors reached the conclusion that the

antilytic action was concerned with the effect which these salts had upon complement. Arkin expressed the view that substances like lactic acid, in certain concentrations, prevent complement from combining with amboceptor in the hemolytic reaction. Carbon dioxid, lactic acid, hydrochloric acid, urea, benzoic acid, tartaric, acetic acid, acetone, ether, chloroform, and hydrazin sulphate were observed by Sherwood to deflect or to destroy the hemolytic complement in certain concentrations. This author also noted that the zone of concentrations in which complement is fixed very nearly approximates and in many cases coincides with the weakest dilutions producing hemolysis, and may extend down to include a few concentrations which are unable to produce hemolysis.

## TECHNIC

The method employed in the examination of the altered sera was that of the routine Wassermann technic used in our laboratories. Standard amounts of complement, amboceptor, sheep cell suspension, patient's serum and antigen were used in all instances. By employing a standard technic (Haythorn) and one which had proved reliable over a period of years, it was possible to compare our results, after altering the reaction of the serum, with the result obtained with the unadulterated serum examined by the same technic. A 2.5 per cent suspension of sheep's red blood cells was used as the hemolytic indicator in the presence of a unit of antisheep amboceptor and two units of 1:10 fresh guinea-pig serum. The patient's inactivated serum was used in an amount of 0.2 cc. with a standard cholesterolized (0.4 per cent) or acetone insoluble antigen. Each serum was inactivated and tested for the presence of syphilitic antibody at the time of conducting the tests to be outlined. The material which we wish to present in this paper has chiefly to do with the examination of negative sera. However, we have obtained some very interesting results with positive and anticomplementary sera which tend to indicate that it is possible, under given conditions, to alter the reaction of the above mentioned qualities of serum. We prefer, however, to discuss these reactions at another time.

The reaction of the serum was altered by the addition of acetic acid. A 1:1500 dilution of the glacial acid in doubly distilled water was the one found most favorable. This dilution of acid was found to be hemo-

lytic for 1 cc. of the standard 2.5 per cent sheep blood cell suspension when employed in a sufficiently large dose. However, the lytic effect of 0.4 cc. of this dilution of acid for the blood cells was no longer evidenced when the amount of serum (0.2 cc.) employed in the tests was added to the acid and incubated for one-half hour. In this connection, it was found possible to mask the lytic effect of stronger dilutions (1:1000) of acetic acid by means of normal inactivated serum in the amount of 0.2 cc. Further, it may be mentioned that the standard unit of blood cells was resistant to as much as 0.2 cc. of the 1:1000 dilution of acid in the absence of serum. Although the dose of acid could be increased beyond a point where the lytic effect was not held in check, it may be stated that the largest amount of acid used in these tests was not hemolytic in the presence of serum.

The protective effect of antigen for the standard unit of blood cells against the lytic effect of acetic acid was also studied. It was found to exert an antilytic effect upon the acetic acid for sheep's blood cells, although the antilytic effect was not constant. There were zone phenomena developed which showed the antilytic action to be irregular and not uniform in character. This finding was contrary to that for serum, the antilytic effect of which was found to be uniform and constant when the dose and concentration of acid were held within certain limits.

A combination of serum and antigen showed a uniform and constant antilytic action upon the lytic effect of acetic acid which was comparable to the antilytic action of serum alone when the same restrictions regarding the acid were observed.

With the information that mixtures of acid, serum and antigen were not lytic for the standard unit of sheep's red blood cells it was decided to determine whether the complex formed in such mixtures would fix complement. The dilution of acid selected was that of 1:1500 concentration as it was found to be satisfactory not only because its lytic action could be controlled effectively but also because it was found to fall within a zone where complement fixation-like reactions could be obtained with regularity. Under the prescribed conditions the lytic action was nil, whereas the stronger 1:1000 dilution exhibited too marked lytic powers. Again weaker dilutions (1:2000), although much less lytic than 1:1500 dilutions, were found incapable of giving regular evidence of complement inhibition. This feature of finding the hemolytic zone so closely related to the zone exhibiting the most frequent evidences of a complement fixation-like reaction is interesting.

The volume of material in all tubes was 2.5 cc.

TABLE 1

| | AMOUNT 1:1500 $CH_3COOH$, cc. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 |
| Complement units | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Antigen, cc | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Results: | | | | | | | | |
| Ser. 17 (Feb. 17–22) (4 days old) | NH | NH | NH | NH | NH | NH | NH | NH |
| Ser. 3 (Feb. 17–22) (4 days old) | NH | NH | NH | NH | NH | NH | NH | NH |
| Ser. 15 (Feb. 17–22) (4 days old) | NH | NH | NH | NH | NH | NH | NH | NH |
| Ser. 10 (Feb. 22–22) | 50 | 50 | 25 | NH | | | | |
| Ser. 10 (after 24 hours) | 25 | 25 | –NH | NH | NH | NH | | |
| Ser. 17 (Feb. 22–22) | 90 | 75 | 25 | 25 | | | | |
| Ser. 17 (after 24 hours) | 90 | 75 | 25 | 25 | –NH | –NH | | |
| Ser. 11 (Feb. 22–22) | 50 | 50 | 25 | NH | | | | |
| Ser. 11 (after 24 hours) | 25 | 25 | –NH | NH | NH | NH | | |
| Ser. 16 (Feb. 22–22) | 75 | 75 | 50 | 50 | | | | |
| Ser. 16 (after 24 hours) (0.1 cc. serum used) | 90 | 90 | 75 | 50 | 50 | 25 | | |
| Ser. 30 (Feb. 22–22) (24 hours old) | 75 | 50 | 25 | –NH | | –NH | | |
| Ser. 20 (Feb. 22–22) (24 hours old) | 75 | 75 | 50 | 50 | | 25 | | |
| Ser. 7 (Feb. 24–22) (24 hours old) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Ser. 14 (Feb. 24–22) (24 hours old) | | 100 | | 100 | | 100 | | NH |
| Ser. 8 (Feb. 24–22) (24 hours old) | | 100 | | 100 | | 100 | | 50 |
| Ser. 4 (Feb. 24–22) (24 hours old) | | 100 | 100 | 100 | 100 | 100 | 75 | 50 |
| Ser. 20 (Feb. 24–22) (24 hours old) | | 100 | | 100 | | 75 | | NH |
| Ser. 5 (Feb. 24–22) (72 hours old) | | 90 | | 50 | | –NH | | –NH |
| Ser. 6 (Feb. 24–22) (72 hours old) | | NH | | NH | | NH | | NH |
| Ser. 19 (Feb. 24–22) (72 hours old) | | 50 | | –NH | | NH | | NH |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pooled serum (Feb. 24–22) (7 days old) | 100 | 100 | 100 | 100 | 90 | 90 | 75 | 50 |
| Ser. 16 (Mar. 1–22) (24 hours old) | | 100 | | 90 | | –NH | | NH |
| Ser. 9 (Mar. 1–22) (24 hours old) | | 100 | | 25 | | NH | | NH |
| Ser. 18 (Mar. 1–22) (24 hours old) | | 100 | | 100 | | 100 | | 75 |
| Ser. 5 (Mar. 1–22) (48 hours old) | | –NH | | NH | | NH | | NH |
| Ser. 25 (Mar. 1–22) (48 hours old) | | NH | | NH | | NH | | NH |
| Ser. 9 (Mar. 10–22) (72 hours old) | | NH | | NH | | NH | | NH |
| Ser. 12 (Mar. 10–22) (72 hours old) | 25 | NH | NH | NH | NH | NH | NH | NH |
| Controls without antigen | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Controls | 0.05 cc. acid + R. B. C. NH | 0.1 cc. acid + R. B. C. NH | 0.2 cc. acid + R. B. C. NH | 0.3 cc. acid + R. B. C. NH | 0.4 cc. acid + R. B. C. 100 | R. B. C NH | | |

*Note:* Controls for each serum without antigen and without acid showed complete hemolysis.

SERA

Gradually increasing doses of a 1:1500 dilution of acetic acid were added to 0.2 cc. of inactivated normal serum and two units of complement. These mixtures were incubated in the water bath for one-half hour at 37.5°C. At the end of this time 1 cc. of a standard 2.5 per cent sheep's red blood cells and a unit of amboceptor were added separately to each tube. Controls without antigen, acid and serum, as well as blood controls were used.

Table 1 illustrates the results. The figures represent the grade of hemolysis.

A survey of table 1 shows that the reaction of a serum is more easily altered as it becomes older, even when repeatedly inactivated. The degree of change is very marked with some specimens in that these have been converted to a strong degree of positiveness. In no instance did any of these sera exhibit anticomplementary action at the time they were examined. This is evidenced from the controls, when in the absence of antigen complete hemolysis was obtained. It was further determined by the controls that the amount of acid used was not responsible for the clearing of the controls which contained no antigen. These doses of acid were not hemolytic save in the one instance. The amount referred to, namely, 0.4 cc. of a 1:1500 dilution always caused hemolysis. However, in the tests we find that the tube which holds this amount often showed no hemolysis. From what we have noted previously, 0.2 cc. of patient's serum is sufficient to mask the lytic effect of acid and the combination thus formed will fix complement. It will also be observed that amounts of acid which give fixation of complement do not hinder the action of complement in producing hemolysis in the controls. A further interesting feature is the difference in the appearance of the hemolysis caused by acid alone with red cells and acid plus serum, complement, amboceptor and red cells. In the first, the color is of a purplish red hue, while the second type presents a clear bright pinkish red. Examination by spectroscope of these two types showed absorption bands for oxyhemoglobin. The character of the hemolysis in the controls which consisted of serum, acid, com-

plement, amboceptor and red cells was always of the second type and, therefore, in all probability, due to the action of complement and amboceptor.

The order in which the acid was added to the tubes apparently made little difference in the result. It was not necessary to add patient's negative serum or antigen to the acid before adding the complement in order to protect the latter. All manner of orders were tried in making up the acid, serum, antigen, complement complex and a similar result was obtained in all instances. This fact was easily checked in the controls where acid was added directly to complement without deleterious effect to the latter substance.

These facts make it reasonably certain that hemolysis was not due to the effect of acid, and non-hemolysis was not due to the destruction of essential elements which function in the complement amboceptor complex.

Further, it was determined that in order to obtain a result of no hemolysis it was necessary to have a proper dose of acid with serum and antigen. Complement was not used up in the presence of any two of these substances. The rule was to permit the acid and serum to stand together for a time before adding the antigen. Although a distinct precipitate was noted with some sera it was not necessary to have a precipitate occur in order to obtain fixation of the complement. However, it was noted that where a precipitate occurred a strongly positive result was obtained. Whatever the change which brought about the fixation of complement it appeared to be intimately linked with an alteration in the serum which occurred in a variety of grades in different sera as was evidenced by the varying degrees of complement fixation in the presence of antigen.

## SPINAL FLUIDS

From our original observations upon the action of small amounts of acetic acid upon spinal fluids, it was learned that a negative fluid could be influenced to show a positive Wassermann reaction. Therefore, it was decided to study this altered

TABLE 2

| | AMOUNT 1:1500 $CH_3COOH$, cc. | | | | | | | | CELL COUNT | GLOBULIN | WASSERMANN | HOURS OLD | ANTIGEN | AMOUNT SPINAL FLUID USED |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | | | | | | |
| Complement units | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | | | | | | |
| Antigen, cc | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | | | | | | |
| Spinal fluid, cc | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | | | | | | *cc.* |
| Results: | | | | | | | | | | | | | | |
| Polk | 100 | 100 | 50 | 50 | | | | | 0 | 0 | 0 | 72 | Choles. | 0.2 |
| Pooled negative spinal fluid | 50 | NH | NH | NH | NH | NH | NH | NH | 0 | 0 | 0 | 72 | Choles. | 0.4 |
| No. 2 (St. F. Hosp.) | 100 | 50 | NH | NH | | | | | 3 | 0 | 0 | 72 | Choles. | 0.4 |
| B. | 100 | 100 | 90 | 50 | | | | | 0 | 0 | 0 | 72 | Choles. | 0.2 |
| G. | 100 | 100 | NH | NH | | | | | 1 | ± | 0 | 72 | Choles. | 0.4 |
| C. | 100 | 100 | 95 | 25 | | | | | 0 | 0 | 0 | 72 | Choles. | 0.2 |
| D. | 100 | 100 | 100 | 50 | | | | | 0 | 0 | 0 | 72 | Choles. | 0.2 |
| No. 474 (M. H.) | | −NH | | NH | | | | NH | 1 | 0 | 0 | 24 | Lipoid | 0.4 |
| Sp. Fl. (Mag.) | | −NH | | −NH | | | | −NH | 0 | 0 | 0 | 24 | Lipoid | 0.4 |
| No. 472 (M. H.) | | −NH | | NH | | | | NH | 22 | 0 | 0 | 24 | Lipoid | 0.4 |
| L. (St. F.) | | 75 | | NH | | NH | | NH | 2 | ± | 0 | 72 | Choles. | 0.4 |
| D. (St. F.) | | 75 | | −NH | | NH | | NH | 3 | 1+ | 0 | 72 | Choles. | 0.4 |
| McC. (794 Mag.) | | | NH | NH | NH | NH | NH | | 0 | 0 | 0 | 2 | Choles. | 0.4 |
| G. (St. F.) | 100 | 100 | 75 | 50 | | | | | 1 | 0 | 0 | 24 | Choles. | 0.2 |
| B. (St. F.) | 100 | 75 | 75 | 50 | | | | | 2 | 0 | 0 | 24 | Choles. | 0.2 |
| M. (M. H.) | | 100 | | 90 | | 75 | | −NH | 1 | 0 | 0 | 72 | Lipoid | 0.2 |
| R. (M. H.) | | 100 | | 75 | | 75 | | NH | 4 | 0 | 0 | 72 | Lipoid | 0.4 |
| H. (M. H.) | | 100 | | 100 | | 75 | | NH | 1 | 0 | 0 | 72 | Lipoid | 0.4 |
| M. (M. H.) | | 100 | | 100 | | 100 | | NH | 7 | 0 | 0 | 72 | Lipoid | 0.2 |
| N. (M. H.) | | 100 | | 100 | | 100 | | NH | 2 | 0 | 0 | 72 | Lipoid | 0.2 |
| L. (St. F.) | | 100 | | 75 | | NH | | NH | 0 | 0 | 0 | 72 | Lipoid | 0.4 |
| L. (St. F.) | | 100 | | 90 | | 90 | | NH | 2 | ± | 0 | 72 | Lipoid | 0.4 |
| H. (St. F.) | | 100 | | 100 | | 100 | | NH | 0 | 0 | 0 | 72 | Lipoid | 0.2 |

reaction and see with what regularity it occurred. Technic similar in all respects to that employed for the study of sera was used in the examination of the spinal fluids.

The fluids were tested for the Wassermann reaction and were found to be negative at the time of examination. The controls were the same as those used in the study of the sera.

The opportunity to study the reaction in relation to time was not afforded with the spinal fluids as they were obtained from outside sources and usually were more than twenty-four hours old. However, it is evident that a complement

TABLE 3

| | | 1:1500 ACETIC ACID | | | | AGE |
|---|---|---|---|---|---|---|
| | | 0.1 cc. | 0.2 cc. | 0.3 cc. | 0.4 cc. | |
| | | | | | | *hours* |
| Negative serum no. 5 | Lipoid | −NH | −NH | NH | NH | |
| | Cholesterin | −NH | −NH | NH | NH | 48 |
| Negative serum no. 25 | Lipoid | −NH | −NH | −NH | −NH | |
| | Cholesterin | NH | NH | NH | NH | 48 |
| Negative serum no. 18 | Lipoid | 100 | 100 | 100 | 100 | |
| | Cholesterin | 100 | 100 | 100 | 75 | 24 |
| Negative serum no. 9 | Lipoid | 100 | 100 | −100 | NH | |
| | Cholesterin | −100 | 25 | NH | NH | 24 |
| Negative serum no. 16 | Lipoid | 100 | 100 | 100 | NH | |
| | Cholesterin | 100 | 90 | −NH | NH | 24 |

fixation-like reaction can be obtained with spinal fluids by the same methods which were used for the examination of sera. It is interesting to note that the degree of positiveness which can be obtained in spinal fluids with a cholesterolized or a non-cholesterolized antigen is but very little different. Apparently the essential substance for the further preparation of the fluid after the addition of the acid is of a fatty nature and is commonly present in the acetone insoluble lipoids employed in the regular Wassermann technic.

On the other hand, in the examination of sera the same serum when tested with cholesterolized and non-cholesterolized extracts showed, as a rule, stronger degrees of positiveness with the cholesterolized extract. However, occasional sera were encountered which reacted equally well with the non-cholesterolized extract. For comparison with the spinal fluids on this point the results in table 3 are interesting. Sera which were partially positive or only positive with cholesterolized antigen were rather easily converted to full four plus positive reactions.

Sachs and Altman observed that a certain number of cases of syphilis give no reaction when heated serum is used, but in 7 out of 11 cases these authors found that the addition of 1:1000 to 1:2000 normal HCl caused a positive or partial reaction, whereas the addition to normal sera was without effect. The finding of these authors with regard to negative sera is contrary to our experience, at least in so far as acetic acid is concerned. Our observations as to the change in the sign of a negative serum to a positive reaction substantiate those of Cumming.

## EXPERIMENTS WITH GLOBULIN

The mechanism of the reaction observed in the foregoing experiments in all probability involves a complex rearrangement of the individual constituents used in the tests. It has been noted that, although a constant degree of change is not obtained, within certain limits it is possible to demonstrate a type of complement fixation. Attention has been directed to the variations in the degrees of positiveness to which negative sera can be converted. It may be that for certain sera, showing a rather marked alteration from negative to positive, the particular experimental conditions bring about the proper concentrations resulting in the apparently necessary balanced ratio required for a particular serum to show a marked change, whereas another zone of concentration would show an equally decided change on the part of other sera.

Of late much attention has been centered in an attempt to determine the fraction of the serum responsible for the phenom-

enon of complement fixation as it is observed in the Wassermann reaction. The studies conducted by Forssmann are of particular interest in that this author concluded that the Wassermann fixing body was intimately bound with the globulin fraction of the serum. In view of this information and our own observation that a marked change in the sign of a negative serum

TABLE 4

*Sample I*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Globulin in increasing doses, cc. | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | 0.45 | 0.5 | 0.55 | 0.6 |
| Cholesterin antigen | | | | | | | | | | | | |
| Complement 2 units | | | | | | | | | | | | |
| Result | −100 | −100 | −100 | NH | NH | NH | 25 | 50 | 50 | 75 | 90 | 100 |
| Controls without antigen | | | | | | | | | | | | |
| Result | 100 | 100 | 100 | NH | NH | NH | 25 | 50 | 50 | 75 | 90 | 100 |

TABLE 5

*Sample II*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Globulin in increasing doses, cc. | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | 0.45 | 0.5 | 0.55 | 0.6 |
| Cholesterin antigen | | | | | | | | | | | | |
| Complement 2 units | | | | | | | | | | | | |
| Result | 100 | 100 | NH | NH | 50 | 50 | 90 | 100 | 100 | 100 | 100 | 100 |
| Controls without antigen | | | | | | | | | | | | |
| Result | 100 | 100 | NH | NH | 50 | 50 | 90 | 100 | 100 | 100 | 100 | 100 |

occurred when visible particulate matter was seen upon addition of acid, it was decided to study the reaction obtained with the globulin fraction of serum and compare this reaction with that obtained by the use of whole serum.

At first globulin was obtained by diluting 200 cc. of ox blood serum ten times and precipitating with 20 cc. of 1:100 acetic acid. This gave a final concentraton of 1:10,000 acetic acid.

The globulin was then washed in distilled water and redissolved in 10 cc. of 0.1 per cent $Na_2CO_3$ solution. Although the results are not conclusive they are nevertheless very interesting. The following tables show the type of result obtained with two samples of ox blood.

The controls show that the antigen had little or no influence upon the result in that the globulin itself was able to bind complement. It will be noted that the reaction is in the nature of a zone phenomenon with a single maximum wave. Although the conditions of this experiment are somewhat altered by the fact that another reagent, namely, sodium carbonate is introduced, it, however, serves to illustrate the effect of a substance like

TABLE 6

| | AMOUNT GLOBULIN IN INCREASING DOSES, CC. | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 |
| Antigen, cc. | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Complement units | 2 | 2 | 2 | 2 | 2 | 2 |
| Negative serum no. 20 | 100 | 100 | 100 | 100 | 100 | 50 |
| Negative serum no. 19 | 90 | 90 | 90 | 90 | 90 | 90 |
| Negative serum no. 12 | 75 | 75 | 75 | 75 | 75 | 50 |
| Negative serum no. 27 | 100 | 100 | 100 | 100 | 100 | 100 |
| Negative serum no. 29 | 100 | 100 | 100 | 100 | 100 | 100 |
| Negative serum no. 4 | 75 | 75 | 50 | 50 | 50 | 25 |
| Controls for each serum (globulin) without antigen | 100 | 100 | 100 | 100 | 100 | 100 |

globulin upon complement. Attention should also be directed to the fact that the final concentration of acid in the globulin precipitation was less, by more than six times, than the dilution which was found to give uniform results with negative sera.

Globulin precipitated from normal human serum by dilution with distilled water and the addition of 1:1500 acetic acid gave a somewhat different result. The results were not constant and varied with the samples of serum. It was observed here, however, that the controls which contained no antigen did not inhibit the action of complement in causing hemolysis.

The results outlined in the foregoing table would indicate that within a proper zone the globulin fraction of some negative

sera forms a combination with antigen which inhibits hemolysis. Although the degree of inhibition of hemolysis is not as pronounced as that obtained by treating whole serum with acid, there is still a grade of comparison between the reaction obtained with the globulin of negative serum and the whole negative serum. The physical or physico-chemical state of the globulins in whole serum is, no doubt, influenced to a marked degree by the other colloidal constituents.

The difference in the globulin content of normal and luetic serum is one of quantity rather than quality. According to Holker, luetic serum contains an excess of euglobulin which is readily precipitated, a feature commented upon by Vernes. However, this author as well as Porges and others have noted that the difference in precipitation between normal and positive sera by extracts of organs or solutions of lecithins is one of rate, the globulins of luetic serum being very unstable and precipitated at a rapid rate. This point is emphasized in the work of Kiss who showed that antigen exerted its effect primarily by precipitating globulin. Kiss has gone a step further in a study of the effect of various adsorbents on complement, concluding that such substances as kaolin, charcoal and a suspension of *B. typhosus* use up complement according to the law of Freundlich and thus complement fixation is an adsorption phenomenon. Forssmann has called attention to the importance of the globulin fraction in serum and concluded that the Wassermann substance is linked up with the globulin fraction probably in the nature of a lipo-globulin compound.

Modifications in the physico-chemical properties of normal sera suffice, according to Hirschfeld and Klinger, to produce positive complement fixation reactions which are dependent upon alterations in the physical state of the serum globulins. Thus, prolonged agitation of normal sera, contact with kaolin and bacterial suspensions will all bring about such alterations and resultant positive complement fixation reactions.

In our experiments there was used an acid which caused a change in the physical state of the sera evidenced in some instances by the precipitation of visible particulate matter. The

latter sera always showed a marked change from the negative to the positive sign. It is well known that the formation of precipitates, the changes in sizes of particulate matter and, perhaps, electrical conditions, are intimately related to the fixation of complement. However, it was found unnecessary to have a definite visible precipitate to obtain complement fixation, a point which has been stressed by Dean in his study of immunological reactions.

The physical state of the various sera, no doubt, exerted a considerable influence upon the reactions which were obtained. Even minor changes in the physical character of the sera would be sufficient to interfere with the uniformity of a reaction of the type discussed in this study. The question of mutual relation of concentrations with a like suspension equilibrium in all tests exerts an influence upon the quality of the reaction which it is quite impossible to evaluate. In these results there is a type of parallelism with those obtained in the regular Wassermann technic in that different grades of complement fixation are obtained with different sera. Generally speaking, in the Wassermann reaction this is looked upon as due to the amount of Wassermann antibody present in the various samples of luetic serum. Even here, however, the trend of investigation points only to a quantitative difference in the concentration of similar substances in the blood in syphilis and in health.

It was found that acetic acid of a certain concentration served best to give fixation of complement in the presence of antigen and a normal serum. It may be that within this range a certain pH was established which was most favorable for the reaction. It is interesting that the presence of antigen was necessary in the mixture to cause the reaction of "no hemolysis" and it apparently made no difference when the antigen was added, although the results were more uniform when a mixture of acid, serum and antigen stood for fifteen minutes or so prior to the addition of the complement. It may be, if as suggested by Warden that fats lower surface tension, that a surface condensation of complement was permitted depending in amount upon the character of the complex created by the treatment

with acetic acid which substance belongs to the neutral fatty acid series. The electrical potential established by the amounts of acid used probably produced some change in the physical state of the globulins so that complement in the presence of antigen was fixed. Not only must the effect of the acid itself be considered but also the almost inconceivable activity of those very unstable compounds, the phosphatids, which are the active principles of antigen. Work upon the question of the isoelectric points for the artificial complement fixation and the precipitation of globulin is in progress.

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# THE INHIBITION ZONE IN PRECIPITIN REACTIONS WITH THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

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## INTRODUCTION

In 1912 Dean (1) called attention to the importance in precipitin and complement fixation reactions of the quantitative relations between antibody and antigen. He found in precipitin reactions that to obtain maximum precipitation from any quantity of immune serum one must employ the optimal or equivalent quantity of antigen. An excess of either antigen or antibody caused a retardation of the precipitin reaction, and if the excess was great inhibition of precipitation occurred with the result that the visible amount of precipitate was lessened. In complement fixation tests Dean found that the matter of optimal or equivalent proportions was of even greater importance. The proportions of antigen and antibody which produce the most effective precipitation are not, as a rule, those which produce the greatest fixation of complement. For any quantity of serum there are, therefore, two quantities of antigen: one quantity which is optimal for precipitation, and the other which is optimal for complement fixation. The antigens employed by Dean were egg white, horse serum, goat serum, human serum, monkey serum, and saline emulsions of *B. typhosus*. The antisera were prepared by the injection of animals with these materials.

Opie in 1923 (2) while studying antigen-antibody relations in precipitin tests encountered the phenomena described by Dean, and concluded that the "inhibition zone," which occurs when antigen is present in excess, is caused by the solvent action of

the excess of antigen. Opie used horse serum, donkey serum, and crystalline egg albumen as antigens.

The above observations were all made on reactions which involved the interaction of immune serum and true antigens, that is, of antibody and substances which give rise to the development of antibody when injected into animals.

Dochez and Avery in 1917 (3) described the soluble specific substance of pneumococcus. This is a bacterial derivative which, when mixed with specific antipneumococcus antiserum, causes precipitation. However, it is not, strictly speaking, antigenic, since it does not give rise to the development of antibodies (4).

The nature of this substance is, briefly, as follows: It was shown by Dochez and Avery to occur in filtrates of pneumococcus cultures and in the body fluids of experimentally infected animals and of patients suffering with acute lobar pneumonia. The substance is type specific, thermostabile, precipitable by alcohol or acetone, non-dialyzable, and not digested by trypsin. Avery and Heidelberger (4, 5) have recently studied this substance and have found that it consists mainly of a carbohydrate, a polysaccharide or a substance intimately associated with a polysaccharide. It is highly reactive when mixed with the homologous type of antipneumococcus serum, and is type specific. Although it reacts with antipneumococcus serum of the homologous type in dilutions as great as 1:5,000,000, the substance is not, strictly speaking, antigenic. Prolonged immunization of rabbits with this material does not result in demonstrable antibody formation.

The substance is, then, a specifically reactive derivative of pneumococcus, and is, in purified form, practically free of nitrogenous and lipoid material. Since, on chemical analysis, it is a much less complex substance than the antigens usually employed in specific serum precipitations, and since it is not antigenic, it seemed of interest to investigate its behavior in varying concentrations of antipneumococcus serum, and to determine whether there exists between the antibacterial serum and the soluble specific substance an optimum ratio for precipitation. If this should prove to be the case, it would indicate that the inhibition zone encountered by previous investigations in precipitin reactions, may be due to the influence of the actual

reacting substance of the more or less complex antigens employed by them. Parker (6) using "residue antigens" of pneumococcus (material similar to if not identical with the specific soluble substance), filtered saline extract of typhoid bacilli and horse serum as "antigens," studied antigen-antibody relations with respect to complement fixation, and confirmed Dean's observations on the importance of the quantitative relationships between antibody and reacting substance.

## MATERIALS

*Specific soluble substance.* This was furnished by Drs. Avery and Heidelberger and their note on its preparation and analysis follows.

Autolyzed cultures of type II pneumococcus in phosphate broth were concentrated to $\frac{1}{12}$ volume and precipitated with 1.2 volumes of alcohol. The precipitate was centrifuged at high speed. The thick compact middle layer which results from centrifugation contains the soluble specific substance. By repeated fractionation of this material with alcohol or acetone, first in neutral and then in dilute acetic acid solution, followed by repeated fractional precipitation with ammonium sulfate and final dialysis, a highly purified preparation was obtained. By this method about 1 gram of the substance is obtained from each 75 liters of culture used. The substance is amorphous and yields a viscous solution in water. A 1 per cent solution gives no biuret test, yields no precipitate with phosphotungstic acid, mercuric chloride, or neutral lead acetate, gives a faint haze with tannic acid, and is precipitate by boric lead acetate. At a dilution of 1:5,000,000 it gives the Molisch reaction. $[\alpha]_D$ is $+58.7°$; N, 1.2 per cent; P, trace; S, none; C, 46.2 per cent; H, 6.1 per cent. Hydrolysis yields 79 per cent of reducing sugars, of which glucose was identified by the melting point and optical rotation of its phenylosazone.

*Serum.* Antipneumococcus serum, type II, obtained by immunizing a horse first with killed, and finally with living cultures of pneumococcus type II.

*Buffer mixtures.* The buffer mixtures[1] consisted of a solution

[1] The buffer solutions were prepared by Dr. A. Baird Hastings

of 0.05 M $KH_2PO_4$, 0.05 M $H_3BO_3$, and 0.05 M $C_3H_4(OH)(CO_2H)_3$. Samples of this mixture were titrated electrometrically with NaOH to pH 5, 7, and 9.

## METHODS

The various dilutions of immune serum were made in normal salt solution or in the buffer mixture at the desired pH. The soluble specific substance was dissolved in normal salt solution at the concentration desired (by weight) and dilutions of this were made by the addition of various amounts of salt solution or of the buffer mixtures. Separate pipettes were used in making all dilutions. The serum dilutions were made in both salt solution and the buffer mixtures. Five-tenths cubic centimeter quantities of the different dilutions of the reacting substance were added to 0.5 cc. quantities of the different dilutions of the immune serum. The tubes were placed in the water bath at 37°C. for two hours, and at 4°C. for at least twelve hours before the readings were made. The dilutions present after mixture of the immune serum and soluble substance are indicated in the table. The relative amounts of precipitate are shown by plus signs, and the maximum precipitate for each dilution of immune serum is indicated by the letter M.

## EXPERIMENTAL

In the following experiment an attempt was made to investigate the quantitative relationship, for optimum precipitation, between antipneumococcus serum and the soluble specific substance.

*Experiment 1.* Four experiments of this type, differing only in the diluents used for the immune serum and the solution of reacting substance were performed. In the first experiment the immune serum and soluble substance were diluted in physiological salt solution. In the remainder the dilutions were carried out in buffer mixture of pH 5, 7, and 9. Since the results were practically the same in all four experiments, the protocol of only the one made with salt solution as the diluent, will be given. In table 1 the figures represent the final concentrations after mixture of serum and reacting substance.

From table 1 it is seen that for optimum precipitation from any amount of immune serum there exists an optimum concentration of the reacting substance. This is shown graphically in chart 1, where the ordinates represent, in milligrams, the concentration of soluble specific substance, and the abscissae represent, in cubic centimeters, the concentration of immune serum. It is seen that if the maximum precipitate for any dilution of immune serum is charted, the points fall approximately on a straight line. The ratio, therefore, between concentration of antibody and concentration of reacting substance, under the conditions of this experiment, is practically constant.

TABLE 1

| TYPE II ANTI-PNEUMOCOCCUS SERUM | SOLUBLE SPECIFIC SUBSTANCE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1:40,000 | 1:80,000 | 1:160,000 | 1:320,000 | 1:610,000 | 1:1,280,000 | 1:2,560,000 | 1:5,124,000 | 1:10,240,000 |
| 1–2 | ++++M | ++++ | +++ | +++ | ++ | + | + | + | 0 |
| 1–8 | + | ++ | +++M | ++ | + | + | + | 0 | 0 |
| 1–32 | + | + | + | ++M | + | + | + | + | 0 |
| 1–64 | 0 | 0 | + | + | +M | + | + | 0 | 0 |
| 1–128 | 0 | 0 | 0 | 0 | + | +M | + | ± | 0 |
| 1–256 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

M = Maximum amount of precipitate for this dilution of immune serum.

From table 1 it is seen that this ratio varies only between 1:10,000 and 1:20,000.

When excessive amounts of reacting substance are brought into contact with the higher dilutions of immune serum, an inhibition zone is present, in which little if any precipitation occurs. It seemed of interest to determine whether, by using an excessive amount of reacting substance, such an inhibition zone could be demonstrated in undiluted serum, and if so, to measure quantitatively this inhibiting effect by determining the weights of the precipitates resulting from the mixture. This was done in the following experiment.

*Experiment 2.* Four wide mouth centrifuge tubes, 2 cm. in diameter and 4 cm. long, were thoroughly cleaned, dried in the oven at 110°C. and weighed. Into each tube was placed 1 cc. of antipneumococcus serum, Type II, the same pipette being used for each measurement. One cubic centimeter of varying concentrations of the soluble substance in salt solution were then added to the four tubes of serum. The first tube received 1 cc. of a 1:100,000 solution of soluble substance, the second 1 cc. of a 1:10,000 solution, the third 1 cc. of a 1:1,000 solution, and the

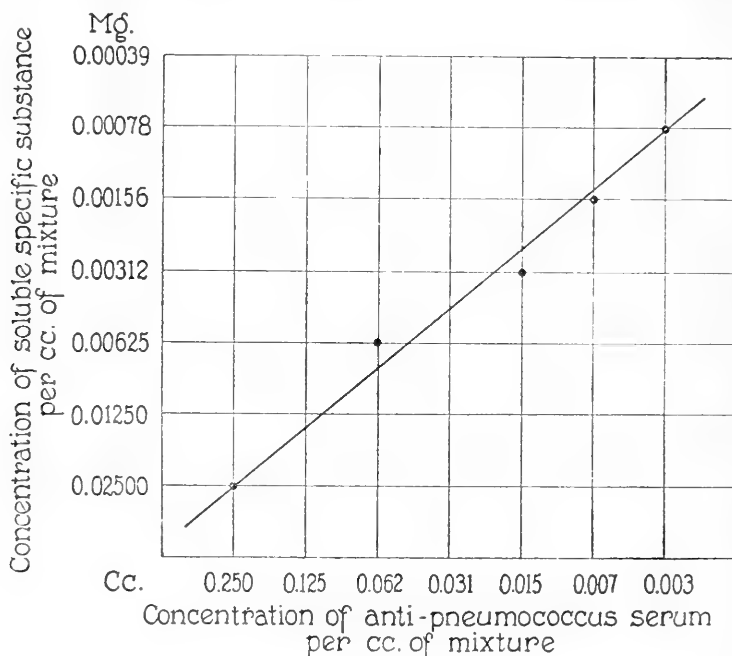


CHART 1

fourth 1 cc. of a 1:100 solution. These solutions were added in the order named using the same calibrated pipette for all measurements. The tubes containing the serum-soluble substance mixtures were placed in the water bath at 37°C. for two hours, and then at 4°C. for eighteen hours. Macroscopic readings of the amount of precipitate were then made and are given in table 2. The four tubes were then centrifuged at high speed for two hours. The supernatant fluid was carefully removed by a pipette and absorbent paper. This was done without

disturbing the tightly packed sediment of precipitate. The tubes were then placed in the oven at 110°C. and dried to constant weight.

In table 2 is given the macroscopic reading of the amount of precipitate in each tube, together with the actual weight of the precipitate.

From table 2 it is seen that when an excess of the soluble substance of pneumococcus is mixed with the homologous type of antipneumococcus serum inhibition of precipitation occurs. Two facts were noted which are worthy of comment in connection with this phenomenon: First, when immune serum and an excessive amount of reacting substance are brought together, a prompt turbidity develops, but this, on standing at room or incubator temperature, rapidly decreases in intensity. An actual solution of the formed precipitate seems to take place. Opie concluded that this occurred when an immune serum was mixed with an excessive amount of its antigen. This conclusion seems also to hold in the case of antipneumococcus serum and the purified reacting substance of pneumococcus. A second observation of interest is that the tube in the inhibition zone which contained the greatest quantity of reacting substance, not only yielded a precipitate macroscopically small, but one which differed in its physical aspects from those of the remaining tubes. Promptly after admixture of serum and soluble substance, a thin, semi-translucent mucilagenous film formed from the turbid mixture and settled to the bottom of the tube. This was in striking contrast to the fluffy, white precipitate occurring

TABLE 2

| TUBE NUMBER | PNEUMOCOCCUS SOLUBLE SPECIFIC SUBSTANCE (TYPE II) | | ANTIPNEUMOCOCCUS SERUM (TYPE II) | | MACROSCOPIC PRECIPITATE | DRY WEIGHT OF PRECIPITATE |
|---|---|---|---|---|---|---|
| | Amount | Final dilution | Amount | Final dilution | | |
| | *cc.* | | *cc.* | | | *gram* |
| 1 | 1 | 1:200 | 1 | 1:2 | ± | 0.0017 |
| 2 | 1 | 1:2,000 | 1 | 1:2 | +++ | 0.0043 |
| 3 | 1 | 1:20,000 | 1 | 1:2 | ++++ | 0.0033 |
| 4 | 1 | 1:200,000 | 1 | 1:2 | ++ | 0.0014 |

in the tubes containing smaller amounts of reacting substance. The importance of recognizing this fact in interpreting the results of a precipitin test is brought out by a comparison of the actual weights of the precipitates, given in table 2, with the macroscopic quantitative determinations. It is seen that the precipitate in tube 1, in which only a very slight precipitate was visible (±), did not weigh less than that in tube 4, which showed macroscopically a fairly heavy precipitate (++), and that the precipitate in tube 3, which macroscopically appeared to be the maximum of the series, weighed no more than that in tube 2 (+++).

## SUMMARY AND CONCLUSIONS

The observations of Dean and Opie upon the importance, in precipitin reactions, of the quantitative relationship between an immune serum and its antigen, also hold for antipneumococcus serum and pneumococcus soluble specific substance. Within the limits of pH 5 to 9, the reaction at which the precipitin test is performed in buffer mixture has little if any influence upon this relationship. In order to obtain the maximum precipitate from any given quantity of antipneumoccoccus serum, a definite, or optimum amount of soluble specific substance must be employed. The ratio between immune serum and reacting substance is practically constant; when an excess of reacting substance is present, the precipitate is not only decreased in amount, as determined by inspection and weight, but its appearance is altered.

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# OBSERVATIONS ON THE ANAPHYLACTOGENIC PROPERTIES OF RYE POLLEN (SECALE CEREALE)

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That pollens are the cause of a high percentage of cases of hay-fever has been well established for quite a number of years. Elliotson, in 1831, seems to have been the first one to ascribe the attacks of hay-fever to pollen. This was particularly established by Blackley, in 1873, who not only collected the pollens on glycerin plates but also induced attacks of hay-fever in himself and in susceptible patients by inhaling them. He also tested the skin reaction of hay-fever patients by applying the pollen to the scarified skin and induced an attack of hay-fever in himself by injecting pollen extract hypodermically. Dunbar, in 1905 and in subsequent writings, published the results of scientific investigations of the affection which finally forced the acceptance of Blackley's experimentally founded theories.

It was shown later that substances other than pollens are capable of producing the hay-fever syndrome. These substances are usually taken in by inhalation, as exemplified by orris root and the animal epithelia. But even non-antigenic substances, such as peptone and tuberculin (1), have been shown to produce the hay-fever syndrome in susceptible individuals.

In 1906, Wolff-Eisner suggested that hay-fever was an anaphylactic reaction and in 1911, Freeman and Noon reported upon the treatment of hay-fever patients with the extracts obtained from the pollens to which they were sensitive. Since

that time a great deal of work has been done along this line. This was especially stimulated by the results of numerous researches along the line of anaphylaxis, and especially since Meltzer, in 1910, pointed out the analogy of clinical asthma with the condition found in experimental, anaphylactic guinea-pigs.

The anaphylactic theory of hay-fever has not been universally accepted because of a number of objections to it. These have been pointed out by many observers, especially by Coca (2). Suffice it to say at this time that the chief objection to it was the fact that many experimental observers in working with the pollen extracts failed to find in them antigenic properties.

Since it has been shown that the offending substance in the pollen is of a protein nature, it was difficult to explain this inability to render guinea-pigs hypersensitive to them. In a careful review of the literature to date of writing (April, 1923) I was unable to find any reports of successful sensitization of experimental animals to the extracts of pollens, with the exception of a brief report by J. T. Parker (3). This author employed the Dale method and reported positive results obtained in guinea-pigs injected with ragweed pollen.

While conducting experiments on anaphylaxis at the Rudolph Virchow Krankenhaus, Berlin (Dr. Kurt Meyer, Director), I made observations on the antigenic properties of pollens. Regretfully, ragweed being absent in Europe, I was unable to carry out experiments on that pollen and had to content myself with utilizing the pollens of rye (*secale cereale*) and maize (*zea Mays*).

In this paper I shall present the results of successful sensitization of guinea-pigs to rye pollen.

## PROTOCOLS

The rye pollen was obtained from Schimmel & Co., from Miltelz bei Leipzig. This firm manufactures the Polantin of Dunbar, and is considered to possess very reliable pollen. The pollen was examined microscopically and found to conform to the structure of a known, fresh specimen of the same pollen.

The pollen was extracted with a 2 per cent solution of sodium bicarbonate after having been previously triturated with a pestle in a mortar. The proportion was 1 gram of pollen to 10 cc. of the bicarbonate solution. After being treated in a shaking machine for about thirty minutes the specimens were put in the incubator for about twelve hours. Toluol was used as a preservative.

The solution was then centrifuged and the supernatant fluid passed through filter paper. The toluol was removed before the solution was injected into the animals. The N content of the solution was determined very accurately and found to be 201.6 mgm. to 100 cc., or 2.01 mgm. to 1 cc.

Twenty guinea-pigs were used for the purpose of these experiments. The animals were first injected on February 2, and 9 of them received a second injection five days later. The latter injection consisted of 2 cc. of the filtered extract with the exception of experimental animals numbers 455 to 459 inclusive, which received only 1 cc. These animals, it will be noticed also had only 1 cc. at the first injection. The test injections were made intravenously in all the animals. Further details of the experiments are to be found in the accompanying table.

## REMARKS CONCERNING THE PROTOCOLS

It will be noticed from the above table that 6 guinea-pigs were injected intraperitoneally with 2 cc. each of rye pollen extract containing 0.2 per cent N. Five animals were injected with 1 cc. of the same extract. Three animals were injected with 2 cc. each of the extract but 0.5 gram of the sediment were added so as to include whatever antigens the solvent might not have dissolved. Four other animals had 2 cc. of each the pollen extract with $\frac{1}{50}$ lethal dose of diphtheria toxin, in order to determine whether the toxin will render them more sensitive to the anaphylactogenic substance. By consulting the table one can see that all the animals have become sensitized, and those that received the extract only were as sensitive as those that had the addition of either sediment or diphtheria toxin.

The observations on these animals consisted of: (1) determination of the minimal lethal dose, (2) antianaphylaxis, (3) passive anaphylaxis, (4) specificity of reactions, (5) demonstration of specific antibodies.

TABLE 1

*Summary of Protocols*

| GUINEA-PIG NUMBER | WEIGHT | FIRST INJECTION | SECOND INJECTION | TEST INJECTION (ALL ADMINISTERED INTRAVENOUSLY) | SUBSEQUENT TESTS |
|---|---|---|---|---|---|
| | *grams* | | | | |
| 1 | 240 | 2/2/23. Intraperitoneally rye pollen extract, 2 cc. 0.2 per cent N | 2/7/23 (5 days later). Same amount as before | 2/23/23 (3 weeks later). 1 cc. intravenously of rye pollen solution 0.2 per cent N. Death in 3 minutes preceded by typical symptoms of anaphylaxis autopsy: characteristic findings of anaphylaxis | Gross *pathological* findings: Heart still beating, heart block present. Pulmonary emphysema, intense. Marked delay of coagulation of blood |
| 2 | 240 | Same | Same | 3/23/23. 0.3 cc. intravenously. Very ill. Temperature 35.4°C. Characteristic itching, diarrhea, prostration, gradual recovery | 2/24/23. 1 cc. of same pollen extract injected intravenously. No reaction. Temperature 39°C. |
| 3 | 240 | Same | Same | 0.25 of same solution. No symptoms. Temperature 38.8°C. | |
| 4 | 240 | Same | Same | 0.5 cc. of same solution. After 2 minutes, convulsive spasms, paraplegia, exhaustion. Temperature 35°C. Profoundly ill for about 8 hours, then recovered gradually | 2/24/23. 1 cc. of same rye pollen extract. Slight reaction. Itching, diarrhea, Temperature 36°. Recovered within two hours |

| | | | | | |
|---|---|---|---|---|---|
| 5 | 240 | 1 cc. of same solution. | 1 cc. of same solution. | 1 cc. of same solution. After 1 minute arched body, convulsions, violent rhythmical extension movements followed by death within 3 minutes. Autopsy: Typical findings | Pathological findings: Pulmonary emphysema. Delay of coagulation of blood. Binding of complement. Heart still beating |
| 6 | 250 | Same | Same | 1 cc. of Berkefeld filtrate of same solution. Typical anaphylactic death within 3 minutes. Autopsy: Typical findings | Pathological findings: Same as above |
| 7 | 250 | 1 cc. of above solution intraperitoneally | Same | 1 cc. Berkefeld filtrate as above. Characteristic scratching of nose, violent convulsive coughing, spasms, profoundly ill. Temperature 34.4°C. Paraplegia. Died 3 hours later. Autopsy: Typical findings | Pathological findings: Same as above |
| 8 | 250 | Same | Same 2/20/23. 2.5 cc. blood withdrawn for precipitin tests | 0.75 cc. rye pollen extract 0.2 per cent N. Scratching of nose, coughing, diarrhea, spasms, prostration. Temperature 35°C., gradual recovery | Next day 2 cc. pollen extract injected intravenously. No reaction |

TABLE 1—*Continued*

| GUINEA-PIG NUMBER | WEIGHT | FIRST INJECTION | SECOND INJECTION | TEST INJECTION (ALL ADMINISTERED INTRAVENOUSLY) | SUBSEQUENT TESTS |
|---|---|---|---|---|---|
| | *grams* | | | | |
| 9 | 250 | Same | Same 2/20/23. 2.5 cc. blood withdrawn for precipitin tests | 0.75 cc. pollen extract as above. Typical anaphylactic symptoms and death within two minutes: Typical findings | Pathological findings: Same as above |
| 10 | 250 | 2 cc. of above extract + 0.2 gram of sediment injected intraperitoneally | No further injections | 0.5 cc. pollen extract as above. Spasms, convulsions, arched back, violent movements of inspiratory muscles. Death in two minutes. Autopsy: Typical findings | Pathological findings: Same as above |
| 11 | 250 | Same | None | 0.1 cc. of above extract. Scratching of nose and ears, diarrhea, spasms, prostration. Temperature 35°C. Appeared very ill for 6 hours, then gradually recovered | |
| 12 | 250 | Same | None | 2/22/23. Bled for purposes of producing passive anaphylaxis. Dead | Pathological findings: Negative. Precipitin tests: negative. Complement fixation: negative |

| | | | | | |
|---|---|---|---|---|---|
| 13 | 250 | 2/3/23. 2 cc. of same pollen extract + 1/50th lethal dose diphtheria toxin | None | 2/23/23. 0.2 cc. horse serum + 0.8 cc. physiological salt sol. No reaction. Temperature 38.8°C. | |
| 14 | 250 | Same | None | 0.75 cc. rye extract (0.2 per cent N). Violent spasms, coughing, respiratory spasm, dyspnea, prostration. Death within 2 minutes. Autopsy: Typical findings | Pathological findings: Pulmonary emphysema; heart block, delayed coagulation, binding of complement |
| 15 | 250 | Same | None | 2/22/23. 4.5 cc. blood withdrawn for precipitin tests, etc. | Precipitin tests: Negative. Specific complement fixation: Negative. |
| 16 | 250 | Same | None | 1 cc. Maize Berkefeld filtrate (0.2 per cent N). No reaction. Temperature 38.8°C. | Next day injected with 1 cc. rye pollen intravenously. Death within two minutes with typical anaphylactic phenomena |
| 17 | 250 | 2 cc. rye pollen extract (0.2 per cent N). | None | 0.5 cc. rye extract. Scratching, spasms, prostration and diarrhea. Temperature 35.5. Death in 15 minutes. Autopsy: Typical findings | Pathological findings: Same as above |

TABLE 1—*Concluded*

| GUINEA-PIG NUMBER | WEIGHT | FIRST INJECTION | SECOND INJECTION | TEST INJECTION (ALL ADMINISTERED INTRAVENOUSLY) | SUBSEQUENT TESTS |
|---|---|---|---|---|---|
| | *grams* | | | | |
| 18 | 250 | Same | None | Berkefeld filtrate of above 0.75 cc. rye extract. Intense dyspnea. Violent respiratory spasms. Death within two minutes. Autopsy: Typical findings | Pathological findings: Same |
| 19 | | None | None | 2 cc. intravenously of rye pollen. No reaction | |
| 20 | 250 | 2/22/23. Injected intraperitoneally with 5 cc. serum obtained from guinea-pig 12 | None | 2/23/23. 1 cc. Berkefeld filtrate rye pollen intravenously. Intense dyspnea, respiratory spasm, convulsions and death within two minutes. Autopsy: Typical findings | Pathological findings: Heart still beating, heart block, pulmonary emphysema, delayed coagulation, binding of complement |

### *Determination of the minimal lethal dose*

In the guinea-pig this was found to be 0.5 cc. of the extract or approximately 1 mgm. of N. 0.75 cc. proved fatal in 3 of the 4 animals injected with it (100 per cent). The extract passed through the Berkefeld filter proved to be of about the same "toxicity" as the one passed through filter paper.

### *Antianaphylaxis*

As will be noticed from the protocol tables, guinea-pig 4 that recovered after a severe reaction following the injection of 0.5 cc. of the extract had but a slight reaction following the administration of 1 cc. on the next day. The injection of the latter dose, as will be noticed, was fatal to all the other animals upon which it was tried. Similarly, guinea-pig 8 that recovered following an injection of 0.75 cc. of the extract did not succumb to the injection of 2 cc. administered on the following day. These two observations show conclusively that the phenomenon of antianaphylaxis became established.

### *Passive anaphylaxis*

Guinea-pig 20 was injected intraperitoneally with 5 cc. of serum obtained from animal 12. Next day the animal was injected with 1 cc. of rye pollen extract intravenously. Typical anaphylactic death resulted within two minutes. A normal control animal exhibited no untoward symptoms whatever from a dose twice that size.

### *Specificity of reaction*

Guinea-pig 16 was injected with 1 cc. of maize pollen extract without any untoward symptoms while 1 cc. of rye pollen extract injected on the following day resulted in a typical anaphylactic death.

### *Specific antibodies*

Precipitins and specific complement-fixing bodies were searched for in 4 animals but with negative results.

## SUMMARY

This paper is based upon observations made on 20 guinea-pigs to determine the anaphylactogenic properties of the pollen of rye (*secale cereale*). The pollen was obtained from a reliable source and microscopically showed the characteristic features of that pollen. It was extracted with a 2 per cent solution of sodium bicarbonate and the extract contained 2.01 mgm. N to 1 cc. Nine animals received only one sensitizing injection. Four animals received an initial injection of 2 cc. (4.02 mgm. N) and the same amount was repeated five days later. Five animals received an initial injection of 1 cc. (2.01 mgm. N) and a repetition of the same was given three weeks after the primary injection. While normal guinea-pigs were found to survive, and without symptoms, 2 cc. of the rye pollen extract, the sensitized guinea-pigs invariably succumbed to injection of 1 cc. of the extract (100 per cent). Of 4 animals injected with 0.75 cc. of the extract, 3 (75 per cent) succumbed within two minutes of injection exhibiting typical anaphylactic symptoms, while 1 gradually recovered following a severe anaphylactic reaction. Dosages of 0.5 cc., or less, invariably gave rise to marked anaphylactic symptoms, but the animals gradually recovered. These animals invariably showed a subsequent increase of tolerance as is evidenced by their surviving the injection of 1 cc. of the extract on the following day.

Passive transfer was accomplished from the sensitized guinea-pig to a normal one. The injection of 1 cc. of the rye extract intravenously on the day following an intraperitoneal injection of 5 cc. of the sensitized animal's serum, proved fatal within two minutes.

## CONCLUSIONS

As a result of the above experiments it seems safe to conclude that guinea-pigs can be readily rendered hypersensitive to rye pollen.

The experimentally injected animals also show the phenomena of anti-anaphylaxis and also of specificity.

Specific precipitins and complement-fixing bodies were not demonstrated in the animals injected with the rye pollen.

*Note:* I wish to refrain, at this time, from drawing any inferences as to the bearing of the above observations on the symptom complex of hay-fever in man. Further experiments are now being conducted on guinea-pigs and rabbits with the pollen of rye and also of ragweed. Observations on the latter pollen will undoubtedly have a more direct bearing on the human hay-fever question.

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# HUMAN HYPERSENSITIVENESS TO DIFFERENT PROTEINS OF HORSE SERUM[1]

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During the past five years it has been our custom to test for immunity to diphtheria and hypersensitiveness to horse serum all nurses and other adults who were later to go on service in an associated contagious hospital. Among these individuals there was encountered one whose endermal reaction to normal horse serum was unique, and is here reported because of its obvious and interesting relation to a not uncommon phenomenon of serum disease—that of recurrent rashes following a single injection of unconcentrated serum.

The subject is a healthy woman, born in 1897, who, in 1905, received a small prophylactic injection of diphtheria antitoxin. The source of the serum cannot be traced, but since concentrated preparations were in the experimental stage until Gibson's investigations in 1906 we may securely assume that whole horse serum was used. No symptoms whatever followed this injection. The subject is of a hypersensitive family if this is evidenced by maternal eczema, fraternal and sororal urticaria, asthma in a maternal second cousin, and personal urticaria in childhood. This allergic inheritance may well be an important factor in the peculiar type of hypersensitiveness now to be described.

On March 3, 1920, she received on the volar surface of the forearm an endermal injection of 0.1 ml. of normal horse serum diluted 1 in 10. In about twenty minutes an itching wheal developed, reached its maximum size in about ten minutes, then measuring 1.2 cm. in diameter with a 3 cm. zone of erythema, and subsided in an hour.

[1] Presented at the tenth annual meeting of the American Association of Immunologists at Boston, Mass., March 30, 1923.

Five hours after the test was made, there appeared at the same site a larger itching wheal 1.7 cm. across with redness 3.5 x 4.5 cm.; this reaction disappeared entirely in less than two hours.

Some twelve hours after the test, intense itching suddenly called the young woman's attention to her arm and at the same site there was observed another wheal and reddened area slightly larger than the two preceding reactions. A faintly pink area 6 x 8 cm. was still visible the next morning.

Mechanical irritation, from scratching, was not a factor in the reappearance of the wheals and erythema. Later tests induced the same sequence of reactions.

Naturally, this triple response led us to assume that it was probably due to multiple sensitiveness to horse serum proteins and that of them, albumin, euglobulin and pseudoglobulin, being the three predominant varieties, were most likely to be implicated. Accordingly these three were prepared from normal horse serum by appropriate, six times repeated, fractional precipitations with ammonium sulphate. The fractions thrown down at one-third, one-half and complete saturation were considered respectively as euglobulin, pseudoglobulin and albumin. One should not, of course, consider that these salting out methods give products which are pure and definite entities. It was felt, however, that several repeated partial saturations would in each case reduce accompanying fractions to such a small amount that no cross reactions would be produced in a moderately hypersensitive skin. Prolonged dialysis (forty-eight days) against distilled water was also used to separate the globulins obtained by the primary half saturation of diluted serum. The preparations finally obtained were dialyzed to remove ammonium sulphate, sterilized by Berkefeld filtration, tested for nitrogen content, for ability to react with an anti-horse precipitating serum, and for ability to excite endermal reactions of hypersensitiveness.

The results of these tests are as follows:

*Endermal reactions to separated proteins of horse serum*

| | NITROGEN | TITRE WITH ANTI-HORSE PRECIPITATING SERUM | ENDERMAL REACTION TO 0.05 ML. |
|---|---|---|---|
| | *per cent* | | |
| Normal serum | 0.12 | 1:5120 | Wheal, erythema, and itching in 30 minutes; again in 5 hours; again in 12 hours |
| Pseudoglobulin | 0.03 | 1:160 | Erythema, 3 cm., in 60 minutes; moderate itching; no wheal |
| Pseudoglobulin, D* | 0.2 | 1:1280 | Erythema, 3 cm., in 60 minutes, followed by diffuse swelling |
| Euglobulin | 0.05 | 1:40 | Erythema in 5 hours; maximum, 3 cm., in 10 hours; no wheal, no itching |
| Euglobulin, D | 0.03 | 1:40 | Erythema in 7 hours; wheal, 1.5 cm., with erythema, 4.5 cm., and marked itching in 10 hours |
| Albumin | 0.22 | 1:2560 | Erythema and diffuse swelling beginning in 7 hours; maximum, 3.5 x 5. cm., in 13 hours; no itching |

None of these preparations caused a reaction in normal individuals.

*D = separated by dialysis.

Corroborative tests were made later with a concentrated antidiphtheric serum and an albumin prepared by half saturating diluted serum with ammonium sulphate, adding three volumes of acetone to the filtrate, and dissolving the precipitate in a weak sodium bicarbonate solution (Coca's extracting fluid). The concentrated serum, pseudoglobulin, induced a wheal with erythema and itching in thirty minutes; this subsided but was followed by mild erythema, without wheal or itching, which was most prominent about eight hours after the test. No further reaction appeared. Evidently not all of the euglobulin was removed in the refining process.

The site of the injection of albumin began to show redness after seven hours and was most prominent in thirteen hours; there was no wheal or itching.

It is seen that the first reaction, in point of time, is induced by pseudoglobulin; the second by euglobulin, and the third by albumin. Further, the times at which these reactions appear correspond rather closely with the times at which the three successive reactions develop following a single injection of whole serum. Compared with whole serum, the slightly prolonged intervals between the injection and the appearance of the reactions to the isolated proteins may reasonably be attributed to their diminished solubilities induced by repeated salting out in the process of purification. It is likely, also, that this prolonged manipulation caused enough "denaturation" to account for the abridged character—usually an absence of wheal and pruritis—of the reactions to these fractions. Such an explanation is substantiated by the similar character of reactions engendered by the original lot of whole serum which had been subjected to the "denaturing" influence of protracted storage in the cold. After one year, the wheal, which was a feature of all three successive reactions to fresh serum, had been replaced in the second and third, by a number of small white itching elevations closely surrounding the needle puncture. After two years, the primary wheal still developed, but no urticarial swellings whatever accompanied the erythema of the two following reactions and itching was very slight.

Rabbit serum has produced no endermal response.

A mild endermal reaction was caused by a concentrated (1:50) extract of horse dander. Ordinary exposure to horses has never induced allergic symptoms.

It may therefore be concluded that this triple reaction to horse serum is due to individually specific hypersensitiveness to the three most abundant proteins therein contained, and that, in point of chronologic sequence, the reactions are due respectively to pseudoglobulin, euglobulin, and albumin.

Another individual has recently been found who reacts typically to whole serum in fifteen minutes and again in five hours. Pseudoglobulin causes the early reaction; euglobulin is innocuous; albumin causes a reaction,—wheal, itching, and redness—after five hours.

### RECURRENT SERUM RASHES

Following a single injection of serum into the human body an eruption frequently develops after a variable incubation period; it lasts a few hours or days and then disappears. Sometimes it recurs and a number of instances have been reported in which the rash was observed in two, three or even four distinct, successive periods.[2] It has been estimated that about 6 per cent of patients who develop a serum rash have one or more recurrent attacks. These appear only when whole horse serum is used; following injections of refined antitoxin sera, pseudoglobulin, no recurrences were noted in Wells' series of 9 cases or in the 129 reviewed by Coca.

It was suggested by Goodall that the successive crops of serum rash represent separate reactions to sera, of different horses, which may have been pooled. This explanation is improbable because pooled pseudoglobulins have not been observed to produce recurrent attacks; because serum from one horse has produced recurrent endermal reactions; and because there is more convincing evidence that successive crops are the manifestations of successive appearances, at different time intervals, of sensitiveness to the different serum proteins—the explanation proposed by Dale and Hartley and substantiated statistically by Coca.

There is ample evidence that euglobulin, pseudoglobulin, and albumin are endowed with readily distinguishable antigenic properties. Leblanc (1901) obtained specific precipitins; Dale and Hartley (1916), Kato (1917), Doerr and Berger (1922), and Stern (1922) have observed relative or marked specificity of anaphylactic reactions. All of the recent investigations have disclosed the fact that the three proteins differ considerably in their antigenic "activities." These diminish with increasing solubility in ammonium sulphate solution; euglobulin is the most active fraction, albumin the least active. Larger doses of albumin are necessary to sensitize and to shock guinea pigs than is the case with euglobulin. The latent period before hypersensitive-

[2] Axenow, Daut, Davidson, Goodall, Hartung, Heubner, MacKenzie, von Pirquet and Schick, and others.

ness develops is appreciably longest with albumin. Incidentally, as Coca has shown, precipitin for egg globulin is produced earlier than for egg albumin.

Davidson has recently conducted a detailed investigation of the various forms of serum rash and their relation to the proteins of whole horse serum. He recognizes three kinds of rash, urticarial, morbilliform and circinate; he considers that the diffuse erythematous type is probably due to scarlet fever. The average day of appearance of the rashes following injection is as follows: urticarial, the ninth; morbilliform, the twelfth; circinate, the fourteenth. There being these three types of rash, and three dominant specific varieties of proteins in whole horse serum Davidson concluded that each protein acts specifically in the human body to produce a certain type of rash. His correlation is as follows: Euglobulin produces the urticarial type because in experimental animals sensitiveness to euglobulin develops soonest, and because when concentrated serum is employed, with removal of euglobulin (and albumin) the urticaria is almost totally eliminated. The latter contention may be disputed; Park states that with regard to concentrated serum the later the development of the rash the more likely is it to be urticarial in nature. Sensitiveness to albumin develops latest and therefore probably causes the delayed type of circinate rash. Pseudoglobulin probably causes the morbilliform eruption. Thomson also reports that the circinate rash develops late and is more frequent after a previous urticaria.

Davidson's conclusions are admittedly theoretical and based on obviously circumstantial evidence. The triple skin reaction previously described, however, is direct evidence that man does become specifically hypersensitive to the several horse serum proteins, and constitutes a hitherto missing link in the chain of evidence which strongly supports the theory proposed by Dale and Hartley. The chronologic relations are at variance. Davidson would assign the primary reaction to euglobulin while in my observations the response to pseudoglobulin developed first. However, the relation of individual proteins to individual rashes awaits the testimony of direct experimentation.

It is rather puzzling to find a satisfactory explanation of the sequence of these endermal reactions. Is it to be ascribed to different rates of diffusion of the proteins into the sensitive cells where the reaction takes place? I have found no pertinent data on the diffusibility of these proteins[3] but it seems unlikely that their rates should differ sufficiently to account for a delay of some hours in the development of skin reactions. Such latent periods are not observed when these proteins are brought into contact with specifically sensitive uterine strips.

Is the sequence due to unequal degrees of skin sensitiveness to the three proteins? This again seems to lack adequacy as an explanation because in other examples of hypersensitiveness, notably to pollen, the time necessary for the development of a reactions in a highly sensitive patient is but a little less than when the test is made on a slightly hypersensitive patient. Reactions to pollen extracts may develop a very few minutes sooner to strong than to weak solutions but in the case under discussion the different concentrations of the protein solutions tested do not accord with the sequence of reactions because, although the albumin solution was the strongest of the three, it was by several hours the last to evoke an endermal response.

Perhaps the reactions are not mediated by sessile antibodies already present at the site of injection; the latent period may represent the time necessary for some of the antigen to diffuse into the circulation, be carried to internal antibody reservoirs, there to initiate a mobilization of antibodies which gradually arrive at the site of injection, reach the threshold concentration, and an endermal reaction ensues. Inasmuch as there are well defined differences in the antigenic "activities" of these proteins it may not be unreasonable to assume that the mechanism for mobilizing antibody for albumin may not be geared to as high a speed as that for pseudoglobulin.

One objection to this hypothesis is that when a specific antibody forming mechanism has once been set in motion it responds to further stimulation with a more rapid shedding of antibodies into the circulation. Since the first tests were made on the

[3] Viscosities: albumin $<$ pseudoglobulin $<$ euglobulin (Oswald).

individual under discussion, she has received a number of small doses of horse serum, nevertheless the time intervals between the three reactions have not been appreciably altered.

## SUMMARY

A triple reaction to horse serum has been shown to be due to specific hypersensitiveness, to serum albumin, euglobulin and pseudoglobulin. Thereby the evidence that these proteins are severally and specifically concerned with the manifestations of recurrent serum sickness is strengthened.

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# A HITHERTO UNDESCRIBED PAIR OF ISOAGGLUTINATION ELEMENTS IN HUMAN BEINGS

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Landsteiner (1), in 1901, discovered three human blood groups, and in 1902, Decastello and Sturli (8), pursuing the study with Landsteiner's knowledge and encouragement, found the fourth group. Landsteiner recognized the existence in human sera of two isohemagglutinins, which were designated with the small letters a and b, or (by von Dungern and Hirschfeld (4) $\alpha$ and $\beta$ and two isoagglutinable substances in the corpuscles which were called A and B.

In 1907, Jansky (2, 3) reported the existence of four human blood groups, the relation of the isoagglutination elements being as presented in table 1.

Two years later (1909) Moss (5) also described the four blood groups. Before publishing his paper Moss read Jansky's article, and added a footnote giving Jansky's numbering; but Moss offered another numbering in which the numbers of Jansky's first and fourth groups were reversed.

The Jansky numbering has been adopted on the basis of priority by the American Association of Pathologists and Bacteriologists, the Society of American Bacteriologists, and the American Association of Immunologists as the official numbering of the four blood groups, and it will therefore be used exclusively by us in this paper.

It has long been suspected that there were present in human blood isoagglutination elements other than the original two, and in February of this year, Guthrie and Huck reported the discovery of a third pair of isoagglutination elements, which,

to bring the terminology into conformity with the previously existing one, we will refer to as isoagglutinin c and isoagglutinable substance C. The relation of the exceptional individuals observed by Guthrie and Huck (7) to the four blood groups (Jansky numbering) is shown in table 2.

In the course of a class demonstration of the two original pairs of isoagglutination elements, the serum of one of us (A. F. C.) a group I individual, was absorbed, first with the washed corpuscles of a group II individual (Levine) and then with the washed corpuscles of a group III individual (Sutton). The supernatant fluid, after these absorptions, no longer clumped the corpuscles of the respective group II and group III individuals, but still possessed a vigorous power of agglutinating the corpuscles of a group IV individual (Johnson) which were agglutinable by the serum of both Levine and Sutton.

TABLE 1

| | GROUP I | GROUP II | GROUP III | GROUP IV |
|---|---|---|---|---|
| Serum | ab | b | a | |
| Corpuscles | | A | B | AB |

TABLE 2

| | GROUP I | GROUP II | GROUP III | GROUP IV |
|---|---|---|---|---|
| Serum | ab | b | a | |
| Corpuscles | | A | B | AB |
| Guthrie and Huck | abc | b | c | |
| | | AC | B | |

This observation indicated the presence of another undescribed pair of isoagglutination elements, which we have designated as x (isoagglutinin) and X (isoagglutinable substance). It is evident that the agglutinable substance X is lacking in the group II and group III corpuscles that were used for the absorption, but is present in the group IV corpuscles. The subsequent study was planned to determine whether the x isoagglutinin is present in any group III sera, whether the isoagglutinable sub-

stance is present in group II corpuscles as well as those of group IV, and, finally, in about what percentage of the individuals of the different groups these elements occur.

The technic employed in the study was as follows: To 1 cc. of serum was added, every two minutes, one drop of the washed corpuscular sediment with which the absorption was being carried out; it usually required about 8 drops of the sediment completely to absorb the respective isoagglutinin.

The group III sera were examined merely by absorbing them with L.'s (X negative) corpuscles, and testing the supernatant fluids with L.'s corpuscles, and also with corpuscles, such as J.'s, that possessed the X substance.

The group I sera were at first absorbed with both L.'s and S.'s corpuscles and tested, first with both those corpuscles to prove the completeness of the absorption, and then with J.'s corpuscles. After it was found that the X agglutinable substance is present in some group II corpuscles, these were used instead of J.'s cells to demonstrate the presence of x agglutinin in the group I sera that had been absorbed only with L.'s or some other X negative group II corpuscles.

The presence or absence of X agglutinable substance in group II corpuscles was determined by testing the corpuscles with X positive group I or group III serum from which all a agglutinins had been removed by absorption.

In table 3 are presented the results of the examination of 14 group I sera.

It is seen that 11 of these sera possessed the agglutinin x, while 3 of them lacked it.

In table 4 are shown the results of the examination of 11 group III sera.

It is seen that of the 11 sera, 7 possessed the x isoagglutinin. while 4 lacked it.

The serum of A. F. C., group I, was absorbed with the corpuscles of L., group II, until L.'s corpuscles were no longer agglutinated. The absorbed serum was then tested with 13 other group II corpuscles, and caused agglutination in 11, and no agglutination in 2.

The serum of J. M., group III, was absorbed with the corpuscles of L., group II, until L.'s corpuscles were no longer agglutinated. The absorbed serum was then tested with 9 other group II corpuscles, and caused agglutination in 7, and no agglutination in 2.

Total number of group II corpuscles examined.................... 23
Corpuscles containing agglutinable substance X.................... 18
Corpuscles not containing X.................................... 5

TABLE 3

| GROUP I SERA | UNABSORBED TESTED WITH CORPUSCLES OF | | AFTER ABSORPTION WITH L. AND S. CORPUSCLES TESTED WITH CORPUSCLES OF | | | |
|---|---|---|---|---|---|---|
| | L. | S. | L. | S. | J. | K. |
| 1 (A. F. C.) | ++ | ++ | 0 | 0 | ++ | |
| 2 | +++ | ++ | 0 | 0 | ++ | |
| 3 | ++ | ++ | 0 | 0 | +± | |
| 4 | ++ | ++ | 0 | 0 | 0 | |
| 5 | ++ | ++ | 0 | 0 | ++ | |
| 6 | + | + | 0 | 0 | ++ | |
| 7 | ++ | ++ | 0 | 0 | ++ | |
| 8 | ++ | ++ | 0 | 0 | ++ | |
| 9 | ++ | ++ | 0 | 0 | ++ | |
| 10 | ++ | ++ | 0 | 0 | 0 | |
| 11 | ++ | ++ | 0 | 0 | 0 | |
| 12 | + | + | 0 | 0 | + | ++ |
| 13 | ++ | ++ | 0 | | | ++ |
| 14 | ++ | ++ | 0 | | | ++ |

Total number of group I sera examined............................ 14
Sera containing isoagglutinin x.................................. 11
Sera lacking isoagglutinin x...................................... 3

L. = P. Levine—group II—lacking agglutinable substance X.
S. = H. B. Sutton—group III.
J. = S. Johnson—group IV—possessing agglutinable substance X.
K. = H. Klein—group II—possessing agglutinable substance X.
\+ = agglutination.
0 = no agglutination.

Only 2 group IV corpuscles were available for examination. These were tested with the sera of group I after the sera had been absorbed with the corpuscles of L., group II, and of S. group III, and were found to be agglutinated by the absorbed sera.

The 2 group IV corpuscles were also tested with 11 group III sera, which had been absorbed with corpuscles of L., and agglutination was obtained with 7 of the 11 sera.

In table 5 is given a summary of the findings in the material examined.

Only a few group II sera were examined for the presence of agglutinin x. It is impossible for this agglutinin to exist in the

TABLE 4

| GROUP III SERA | UNABSORBED TESTED WITH CORPUSCLES OF | AFTER ABSORPTION WITH L. CORPUSCLES TESTED WITH CORPUSCLES OF | | | | |
|---|---|---|---|---|---|---|
| | L. | L. | J. | D. | K. | Sp. |
| 1 | ++ | 0 | ++ | | | |
| 2 | ++ | 0 | 0 | | | |
| 3 | ++ | 0 | 0 | | | |
| 4 | ++ | 0 | ++ | | | |
| 5 | ++ | 0 | 0 | | | |
| 6 | ++ | 0 | 0 | | | |
| 7 (J. M.) | ++ | 0 | ++ | ++ | ++ | |
| 8 | ++ | 0 | ++ | ++ | ++ | |
| 9 | ++ | 0 | ++ | ++ | ++ | ++ |
| 10 | ++ | 0 | ++ | ++ | ++ | ++ |
| 11 | ++ | 0 | ++ | ++ | ++ | ++ |

Total number of group III sera examined........................ 11
Sera containing isoagglutinin x................................ 7
Sera not containing isoagglutinin x............................ 4

L. = P. Levine—group II—lacking X.
S. = H. B. Sutton—Group III.
J. = S. Johnson—group IV
D. = Miss Dunn—group IV
K. = H. Klein—group II
Sp. = Miss Sperandei—group II
(J., D., K., Sp.: Possessing X.)
\+ = agglutination.
0 = no agglutination.

serum of a group II individual whose corpuscles carry the agglutinable substance X; but it could be present in the serum of a group II individual whose corpuscles are X negative. Such a finding is, indeed, to be expected, if the well supported inheritance theory of von Dungern and Hirschfeld applies also to the new pair of elements.

Von Dungern and Hirschfeld presented a large mass of evidence pointing to an independent inheritence of the A and the B agglutinable substances as Mendelian dominants. They referred to the hypothetical contrasting characters as Not A and Not B. Ottenberg's modification of this view consists in his identification of the respective agglutinins as the contrasting characters.

TABLE 5

| | GROUP I (SERA) | GROUP II (CORPUSCLES) | GROUP III (SERA) | GROUP IV (CORPUSCLES) |
|---|---|---|---|---|
| Total cases | 14 | 23 | 11 | 2 |
| Containing x | 11 | | 7 | |
| Without x | 3 | | 4 | |
| Containing X | | 18 | | 2 |
| Without X | | 5 | | 0 |

According to this latter conception, the germ plasm constitution of the heterozygous X positive and X negative group II individuals should be respectively

| (X positive) | | (X negative) |
|---|---|---|
| abx | and | abx |
| bAX | | bAx |

Crosses of two such individuals would produce

abx/abx = group I    abx/bAx = group II

| | abx/abx = group I | abx/bAx = group II |
|---|---|---|
| serum— | abx | bx |
| corpuscles— | — | A |

| | abx/bAX = group II | bAX/bAx = group II |
|---|---|---|
| serum— | b | b |
| corpuscles— | AX | AX |

It is seen that when the corpuscles lack the agglutinable X, the agglutinin x should be found in the serum. However, when the serum of L. and that of P. (group II individuals lacking agglutinable X) were tested with the corpuscles of K. and of Sp., both possessing agglutinable X, no agglutination occurred. Agglutinin x was thus absent from both the tested sera. This result merely confirms the general experience that there is no

clear subdivision of any of the four blood groups, although there is some evidence of a slight subdivision. Ottenberg (9), for example, wrote: "Members of this group (group III) sometimes show slight individual irregularities, . . . . the sera occasionally agglutinating the cells of some, but not all, other members of the third group itself." Unger (6) also states: "In the course of performing many thousands of tests preliminary to transfusion, I have occasionally noted that, although the donor and patient are of the same group, when the bloods are tested one against the other, a small number of agglutinated clumps of blood cells will be seen . . . . although their cells fell definitely into groups II or III, their serum gave minor agglutinative reactions against cells of patients of the same group."

The negative finding just described seems to indicate that agglutinable X is not related to agglutinin x as dominant to recessive character.

As we remarked in our preliminary report, the possibility of the X pair of blood elements being identical with that described by Guthrie and Huck must be considered. Dr. Guthrie was kind enough to send us, in excellent condition, the serum and corpuscles of some of the individuals that had been examined by him. Of these specimens, only the corpuscles of H (group II lacking C) and of D. J. (group II possessing C) lent themselves at all to our purpose. We found, it is true, that the H corpuscles lacked also X, and the corpuscles D. J. possessed this substance, but from this outcome of the tests it cannot be learned whether the C and X substances are identical or not. This could only be determined with the aid of the serum of C. T. with which reagent Guthrie and Huck made their original discovery of the C substance. Unfortunately, this serum is practically inaccessible. The only alternative in this situation is to compare the percentage incidence of C in group II as observed by Guthrie and Huck with the incidence of X in group II as we have found it.

The former figure is given by the Baltimore workers on page 132 of the April number of the Johns Hopkins Hospital Bulletin

as one in every 4 or 5 bloods; that is at most 25 per cent. We have found the incidence of X in group II to be more than three times as great; that is (table 5) about 78 per cent. These figures indicate that C and X are different substances.

Guthrie and Huck have proposed designating the individuals possessing the C pair with additional group numbers. This would only further complicate an already much confused subject.

The blood grouping has always had a distinctly practical purpose and use in connection with the operation of blood transfusion, and, since the discovery of the C and X pair of blood elements has caused no subdivision of any of the groups with respect to transfusion, it would be a mistake to use these inconsequential differences within a group as a ground for adding to the number of the groups.

Exceptionally, individuals are encountered whose blood constitution makes it questionable in which of two groups they should be classed. However, no practical advantage can be gained by classing these unusual individuals outside the four groups.

The finding of the X pair, as well as of the C pair, has revealed an unsuspected complexity of constitution of the known groups, without, however, changing their practical mutual relationships with regard to blood transfusion.

## SUMMARY

1. A new pair of isoagglutination elements is described occurring in a high percentage of all the four blood groups.

2. The agglutinin x was found in about 75 per cent of group I sera, and about 60 per cent of group III sera. The agglutinable X was found in about 75 per cent of group II corpuscles and in both of the two group IV individuals examined.

3. The relation of the isoagglutination elements in the four groups, including the C pair of Guthrie and Huck, may be represented according to the Jansky numbering as follows:

| | GROUP I | GROUP II | GROUP III | GROUP IV |
|---|---|---|---|---|
| Serum | abx (c) | b | ax (c) | |
| Corpuscles | | AX (C) | B | ABX |

4. The discovery of the new isoagglutination elements in no way alters the practical status of the four original blood groups.

5. The X pair seem not to be inherited as Mendelian allelomorphs.

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# A STUDY OF THE OCCURRENCE OF THE BLOOD GROUPS AMONG THE AMERICAN INDIANS

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The inheritance of the Landsteiner isoagglutination elements of the human blood has been studied in two different respects.

Von Dungern and L. Hirschfeld (1) and, later, Ottenberg (2) and also Buchanan (3) have investigated the mode of transmission of the elements from generation to generation by the examination of the blood in parents and offspring.

L. Hirschfeld and H. Hirschfeld (4) on the other hand, have studied, in about 9000 individuals, the percentage distribution of the four blood groups in the different human races, and, in this way, these authors have obtained statistical data which seem to provide indirect evidence as to the manner of transmission of the blood elements, and which also offer a new means of studying racial relationships.

The evidence referred to lies in the Hirschfelds' observation that the percentages of "found" I and IV (Jansky) groups in the different races approximate the respective percentages which they calculated from those of groups II and III. This calculation was made on the basis of their assumption that if, as von Dungern and L. Hirschfeld believed, the Landsteiner isoagglutinable elements A and B are transmitted as independent Mendelian dominant characters, their combined appearance in group IV (Jansky) must depend upon the chance mating of individuals of group II (possessing the A substance) with those of group III (possessing the B substance). Conversely, on the same theory of the inheritance, the authors calculated the probable incidence of group I from the chance matings of individ-

uals lacking both A and B as found by subtracting the percentage incidences of these two elements (the sum of II and IV (Jansky) and III and IV respectively) from 100.

The agreement between the calculated percentages and those actually found was not exact, but it was so close that it indicates the correctness of the theory of the inheritance adopted by von Dungern and Hirschfeld and supported by the larger study of Ottenberg.

The more striking outcome of the Hirschfeld's investigation is their discovery that the percentage occurrence of the four blood groups differs distinctly and, in some cases, widely in the different races. This discovery, as we have said, disclosed a new method of studying racial relationships, and, as we had the opportunity during our study of the incidence of serum disease in the American Indian, we have taken advantage of it to investigate the question of the relation of the American Indian race to the northeastern Asiatic races (Chinese, Japanese, Koreans). The making of such an investigation was suggested by the generally credited theory that the American Indian is of Mongolian origin and reached the American Continent by way of the extreme northeastern point of Siberia. Of the Asiatic races, we ourselves were able to examine only the Chinese. However, through Dr. M. Kosakai, we are indebted to Dr. Fukamachi (5) for his study of the Japanese and Koreans, as well as the Chinese, which has just been received.

Our examination of American Indians was made in two series upon full-blooded individuals of many different tribes.

The first series was a short one, comprising 112 Indian students in Haskell Institute, of whom 74 were in group I, 30 were of group II, 7 were of group III, and 1 was of group IV. The percentage occurrence of the four groups was thus 66, 27, 6, and 1.

The second series comprised 862 students (341 in the Chilocco Indian School, Oklahoma; 434 in the United States Indian School at Albuquerque, N. M.; and 87 in Haskell Institute, Lawrence, Kansas). Of these 670 (77.7 per cent) were of group I, 174 (20.2 per cent) were of group II, and 18 (2.1 per cent)

TABLE 1

*Showing the percentage occurrence of the four human blood groups in the different nationalities and races*

| | JANSKY GROUPS | | | | | |
|---|---|---|---|---|---|---|
| | I | | II | III | IV | |
| | O | | A | B | AB | |
| | Calculated | Found | | | Found | Calculated |
| **L. and H. Hirschfeld** | | | | | | |
| English | 47.9 | 46.4 | 43.4 | 7.2 | 3.0 | 3.1 |
| French | 46.6 | 43.2 | 42.6 | 11.2 | 3.0 | 4.8 |
| Italian | 49.5 | 47.2 | 38.0 | 11.0 | 3.8 | 4.2 |
| German | 43.1 | 40.0 | 43.0 | 12.0 | 5.0 | 5.1 |
| Austrian | 42.6 | 42.0 | 40.0 | 10.0 | 8.0 | 4.0 |
| Serb | 42.7 | 38.0 | 41.8 | 15.6 | 4.6 | 6.5 |
| Greek | 43.4 | 38.2 | 41.6 | 16.2 | 4.0 | 6.7 |
| Bulgarian | 42.3 | 39.0 | 40.6 | 14.2 | 6.2 | 5.8 |
| Arab | 47.5 | 43.6 | 32.4 | 19.0 | 5.0 | 6.5 |
| Turk | 39.9 | 36.8 | 38.0 | 18.6 | 6.6 | 7.0 |
| Russian | 44.9 | 40.7 | 31.2 | 21.8 | 6.3 | 6.8 |
| Jew | 44.5 | 38.8 | 33.0 | 23.2 | 5.0 | 7.6 |
| Malagasies | 49.5 | 45.5 | 26.2 | 23.7 | 4.5 | 6.2 |
| Negroes (Seneg.) | 47.6 | 43.2 | 22.6 | 29.2 | 5.0 | 6.6 |
| Annamese | 45.3 | 42.0 | 22.4 | 28.4 | 7.2 | 6.4 |
| Indian | 43.7 | 31.3 | 19.0 | 41.2 | 8.5 | 7.8 |
| **Kilgore and Liu** | | | | | | |
| Chinese (100) | 33.9 | 28.0 | 36.0 | 25.0 | 11.0 | 9.0 |
| **Liu and Wang** | | | | | | |
| Chinese (1000) | 36.4 | 30.0 | 25.0 | 34.0 | 10.0 | 8.5 |
| **Fukamachi** | | | | | | |
| Japanese | 30.6 | 26.8 | 40.9 | 18.4 | 13.9 | 7.5 |
| Koreans | 33.2 | 28.1 | 32.8 | 26.4 | 12.7 | 8 6 |
| Manchus (North) | 34.6 | 26.6 | 26.63 | 38.2 | 8.5 | 10.1 |
| Chinese (South) | 41.4 | 37.4 | 28.6 | 25.7 | 8.5 | 7.3 |
| Chinese (Central) | 38.2 | 31.1 | 37.8 | 24.4 | 6.6 | 9.2 |
| Chinese (all) | 37.6 | 30.9 | 31.2 | 29.3 | 8.4 | 9.1 |
| **C. and D.** | | | | | | |
| Chinese | 35.4 | 29.0 | 32.0 | 29.0 | 10.0 | 9.3 |
| *American Indian* | *78.1* | *77.7* | *20.2* | *2.1* | *one* | *0.4* |

were of group III. No individual of group IV was encountered in this series.

We are prevented from combining the figures of these two series on account of the fact that some of the students in Haskell Institute were inadvertently included in both series.

The difference between the figures of the two series is considerable, yet both results show in common a high percentage of group I with a corresponding low percentage of the other three groups.

Through the kindness of Miss Tsae Kwong, student of medicine in Cornell University, and with the assistance of Ella F. Grove, we obtained the opportunity of examining the blood of 111 Chinese students on the occasion of their annual conference, which was held at Ithaca in the Fall of 1922. Of these, 32 were of group I; 36 of group II; 32 of group III, and 11 of group IV.

Since we made these examinations, we have learned of the two previous studies of Kilgore and Hua Liu (6), who examined 100 Chinese, and of Heng, Liu and Wang (7), who examined 1000 Chinese. In these latter two studies, the Chinese were not grouped in accordance with their geographical origin (North, Central, South) as in the study by Fukamachi.

In table 1, are presented, for the purpose of convenient comparison, the percentages of the occurrence of the four groups in the different races, as found by the different observers.

While the results of the examination of the Indians do not permit very far reaching conclusions, they do give distinct indications in two directions.

In the first place, notwithstanding the striking difference between the percentages found in the Indians and those of all other races, the percentages calculated on the basis of the von Dungern-Hirschfeld assumption are remarkably close to the corresponding observed percentages.

Secondly, the findings indicate no closer relationship of the Indians with the Eastern Asiatic races than with any of the others. This does not deny at all the current view of the origin of the American Indian; it only offers further evidence as to the antiquity of the race as such.

In view of the high proportion of the group I individuals (nearly four-fifths) among the Indians, the possibility must be considered that the Indians became separated from the rest of the human family before the appearance of the isoagglutination elements, and that the existence of those elements in some of the individuals examined is due to an admixture of white blood. The fact that all of the individuals are listed as full-blooded Indians in the Government records does not weigh against this suggestion, because those records cover only the more recent decades, and list, as full-blooded Indians, numerous individuals who betray in their outward characters an admixture of white or negro blood.

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# AGGLUTININS FOR BACTERIUM PULLORUM IN HENS' EGGS

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## INTRODUCTION

In studying the antibodies produced by *Bacterium pullorum* it occurred to the writer that, since the albumin of the egg may be considered physiologically as a secretion, and that antibodies have frequently been detected in secretions, antibodies for *Bacterium pullorum* might be present in eggs from hens known to be infected with this organism.

## EXPERIMENTAL

Eggs were collected from several hens, the serum of which agglutinated *Bacterium pullorum*. The albumin of the eggs of some of these hens was used in place of the serum in making the agglutination test. The results of the preliminary tests were so striking and definite as to indicate the possible use of egg albumin in place of blood serum in detecting infected hens. The advantages of such a method are quite evident and a more careful study of the problem seemed desirable.

For this purpose 16 hens, 10 reactors, 5 non-reactors, and 1 questionable reactor, were isolated and, as far as possible, kept under the same environmental conditions during the experiment. The hens were trap nested and a complete record kept of all eggs laid. The albumin of all eggs laid during the experiment was tested for agglutinins. The agglutinin titer of the blood serum for *Bacterium pullorum* of each hen at the beginning and at the end of the experiment is recorded in table 1.

TABLE 1

*Showing reaction of serum on Bact. pullorum at beginning and end of experiment*

| HEN NUMBER | DATE | SERUM DILUTION | | | | | | | | INCUBATION HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 | 1:1280 | 1:2560 | 1:5120 | 37°C. | Ice box |
| 1 | 7/14 | ++++ | ++++ | ++++ | ++++ | +++ | ++ | | | 16 | |
| | 9/15 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | 24 | 18 |
| 2 | 7/14 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | | | 16 | |
| | 9/15 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 | 24 | 18 |
| 3 | 7/14 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | | | 16 | |
| | 9/15 | ++++ | ++++ | ++++ | +++ | ++ | 0 | 0 | 0 | 24 | 18 |
| 4 | 7/14 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 | 16 | |
| | 9/15 | Not run | | | | | | | | | |
| 5 | 6/15 | ++++ | ++++ | ++++ | ++++ | ++++ | | | | 39 | |
| | 9/15 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | 24 | 18 |
| 6 | 6/15 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | | | 39 | |
| | 9/15 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 | 24 | 18 |
| 7 | 6/15 | ++++ | ++++ | ++++ | ++++ | ++++ | | | | 39 | |
| | 9/15 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 24 | 18 |
| 8 | 6/15 | +++ | +++ | +++ | ++ | 0 | | | | 39 | |
| | 9/15 | ++++ | ++++ | ++++ | + | 0 | 0 | 0 | 0 | 24 | 18 |
| 9 | 6/15 | +++ | +++ | +++ | 0 | 0 | | | | 39 | |
| | 9/15 | ++++ | ++++ | ++ | + | 0 | 0 | 0 | 0 | 24 | 18 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 7/14 | ++++ | ++++ | ++ | 0 | 0 | 0 | 0 | 0 | 16 | |
| | 9/15 | Not run | | | | | | | | | |
| 11 | 7/14 | ++ | + | + | 0 | 0 | | | | 16 | |
| | 9/15 | ++++ | ++++ | ++++ | 0 | 0 | | | | 24 | 18 |
| Controls | | | | | | | | | | | |
| 12 | 6/15 | +++ | + | 0 | 0 | 0 | | | | 39 | |
| | 9/15 | ++++ | + | + | 0 | 0 | | | | 24 | 18 |
| 13 | 7/14 | 0 | 0 | 0 | 0 | 0 | | | | 16 | |
| | 9/15 | ++++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 24 | 18 |
| 14 | 7/14 | 0 | 0 | 0 | 0 | 0 | | | | 16 | |
| | 9/15 | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 24 | 18 |
| 15 | 7/14 | 0 | 0 | 0 | 0 | 0 | 0 | | | 16 | |
| | 9/15 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 24 | 18 |
| 16 | 6/15 | 0 | 0 | 0 | 0 | 0 | | | | 39 | |
| | 9/15 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 24 | 18 |

++++ = complete agglutination.
+++ = marked agglutination.
++ = partial agglutination.
+ = trace of agglutination.
0 = no agglutination.

From the results obtained in table 1 the following birds were considered as having an ovarian infection: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10; while fowls No. 12, 13, 14, 15, and 16 were considered as not being infected. It is evident that hen 11 gave a negative reaction when tested July 14, but a typically positive reaction when tested September 15. Possibly this difference in serum titer is due to the fact that the test made September 15 was incubated for a longer period. However, this seems to have made little difference in the results obtained. In fact, Fowl 3 did not show as high a titer on September 15 when the test was incubated for twenty-four hours in the incubator and eighteen hours in the ice box, as it did on July 14, when incubated only sixteen hours. Considerable variation is noted in the titer of the blood serum of reacting birds. Some are capable of clumping the antigen in high dilution, whereas others cause just sufficient agglutination to be classed as infected.

In addition to studying the agglutinins of the eggs, cultures for *Bacterium pullorum* were also made. Since Gage (1) has shown that a preliminary incubation facilitates the isolation of *Bacterium pullorum* all eggs were accordingly incubated from one to eight days at 37°C. before the tests were made.

## METHOD OF PROCEDURE

Immediately before withdrawing the albumin from the eggs they were placed in a 2.5 per cent solution of phenol for a few minutes. The eggs were removed from the phenol solution with sterile forceps, the large end was flamed and an opening of sufficient size to admit a pipette was made in the shell. One-half of a cubic centimeter of the thin albumin was withdrawn and thoroughly mixed with 1.5 cc. of saline in an agglutination tube. From this were prepared progressive dilutions up to 1:32. To each tube was then added 1 cc. of antigen. The antigen was prepared by mixing the agar slant growth of three strains of *Bact. pullorum* in normal saline. This was heated to 60°C. for thirty minutes and 0.5 per cent phenol added. The tubes were incubated at 37°C. for various lengths of time (sixteen to sixty-five hours) after which they were placed in the ice box and held for twenty to fifty-five hours.

In all, 345 eggs were tested from the 16 experimental birds. The agglutination titers are recorded in table 2.

The results in general seem to show that the albumin of eggs laid by infected hens agglutinates the antigen from a partial to a complete degree in 1:8 dilution; whereas, the albumin of eggs from non-infected hens rarely agglutinates the antigen more than

TABLE 2

*Agglutinative power of egg albumin on Bact. pullorum antigen*

| HEN NUMBER | NUMBER OF EGGS TESTED | DILUTION OF EGG ALBUMIN | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:8 | | | | | 1:16 | | | | | 1:32 | | | | | 1:64 | | | | |
| | | ++++ | +++ | ++ | + | 0 | ++++ | +++ | ++ | + | 0 | ++++ | +++ | ++ | + | 0 | ++++ | +++ | ++ | + | 0 |
| 1 | 8 | 1 | 7 | 0 | 0 | 0 | 1 | 1 | 6 | 0 | 0 | 1 | 1 | 5 | 1 | 0 | 1 | 0 | 1 | 5 | 1 |
| 2 | 29 | 16 | 12 | 1 | 0 | 0 | 14 | 6 | 9 | 0 | 0 | 5 | 13 | 9 | 1 | 1 | 1 | 5 | 11 | 8 | 4 |
| 3 | 5 | 0 | 1 | 3 | 0 | 1 | 0 | 0 | 4 | 0 | 1 | 0 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 1 | 4 |
| 4 | 4 | 0 | 4 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 2 | 0 | 2 |
| 5 | 5 | 3 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 1 | 0 | 1 | 3 | 0 | 0 | 1 | 0 | 4 |
| 6 | 18 | 7 | 6 | 1 | 3 | 1 | 4 | 7 | 3 | 4 | 0 | 3 | 3 | 9 | 3 | 0 | 2 | 3 | 5 | 3 | 5 |
| 7 | 47 | 0 | 0 | 14 | 14 | 19 | 0 | 0 | 11 | 11 | 25 | 0 | 0 | 4 | 14 | 29 | 0 | 0 | 1 | 7 | 39 |
| 8 | 15 | 0 | 7 | 6 | 1 | 1 | 0 | 2 | 9 | 3 | 1 | 0 | 1 | 4 | 8 | 2 | 0 | 0 | 0 | 1 | 14 |
| 9 | 6 | 1 | 4 | 0 | 0 | 1 | 1 | 4 | 0 | 0 | 1 | 0 | 3 | 2 | 0 | 1 | 0 | 0 | 2 | 3 | 1 |
| 10 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| 11 | 16 | 0 | 4 | 7 | 1 | 4 | 0 | 4 | 7 | 1 | 4 | 0 | 3 | 3 | 6 | 4 | 0 | 1 | 1 | 3 | 11 |
| 12 | 30 | 0 | 0 | 10 | 9 | 11 | 0 | 0 | 4 | 11 | 15 | 0 | 0 | 2 | 8 | 20 | 0 | 0 | 0 | 3 | 27 |
| 13 | 49 | 0 | 0 | 9 | 12 | 28 | 0 | 0 | 4 | 14 | 31 | 0 | 0 | 0 | 10 | 39 | 0 | 0 | 0 | 2 | 47 |
| 14 | 26 | 0 | 1 | 4 | 6 | 15 | 0 | 1 | 2 | 6 | 17 | 0 | 0 | 2 | 2 | 22 | 0 | 0 | 1 | 0 | 24 |
| 15 | 35 | 0 | 0 | 3 | 9 | 23 | 0 | 0 | 1 | 6 | 28 | 0 | 0 | 0 | 4 | 31 | 0 | 0 | 0 | 0 | 35 |
| 16 | 51 | 0 | 0 | 6 | 19 | 26 | 0 | 0 | 1 | 20 | 30 | 0 | 0 | 0 | 13 | 38 | 0 | 0 | 0 | 7 | 44 |

a trace in this dilution. This was true in all but 1 case, hen 7. The serum agglutination titer of this hen would indicate that she was a reactor, but the albumin of her eggs failed to clump the antigen. There does not seem to be a correlation between the serum and albumin titers as some birds which show a relatively high serum titer gave a comparatively low albumin titer and vice versa.

To sum up the results given in table 2 the reacting fowls (when serum was used), excluding fowl 7, gave the following titers when egg albumin was used in a 1:8 dilution.

*Summary of table 2*

| FOWLS | EGGS | 1:8 DILUTION EGG ALBUMIN | | | | |
|---|---|---|---|---|---|---|
| | | Degree of agglutination | | | | |
| | | ++++ | +++ | ++ | + | 0 |
| 9 | 91 | 28 | 42 | 13 | 4 | 4 |
| Per cent.......... | | 30.76 | 46.15 | 14.28 | 4.39 | 4.39 |

A summary of the results given in table 2 for fowls reacting negatively to the serum test.

| FOWLS | EGGS | 1:8 DILUTION EGG ALBUMIN | | | | |
|---|---|---|---|---|---|---|
| | | Degree of agglutination | | | | |
| | | ++++ | +++ | ++ | + | 0 |
| 5 | 191 | 0 | 1 | 32 | 55 | 103 |
| Per cent.......... | | 0.0 | 0.52 | 16.75 | 28.79 | 53.92 |

The results seem to show that albumin from the eggs of hens reacting positively to the serum test is capable of clumping a *Bact. pullorum* antigen to a very marked degree and in some cases completely, whereas the albumin of eggs from non-infected hens rarely agglutinates the antigen to this extent.

An examination of table 1 will show that hen 11 was not a reactor when tested on July 14, but when tested two months later gave what should be considered as a positive reaction. This is interesting and may be taken to indicate that she became infected as an adult. Along with the increase in serum titer an increase was noted in the ability of the albumin from her eggs to clump the antigen. This point is shown more clearly in table 3.

It will be noted that considerable time elapsed between the laying of eggs 16 and 17. Just why there should have been a resting period at this time is not known. The presence of a "soft shell' in the case of egg 15 might be significant. Eggs. 17, 18, and 19, all contained bloody material indicating an abnormal condition and in the case of egg 17 plate cultures showed a staphylococcus.

TABLE 3

*Agglutination of Bact. pullorum antigen by albumin of some of the eggs from hen 11*

| EGG NUMBER | LAID | ALBUMIN DILUTION | | | |
|---|---|---|---|---|---|
| | | 1:8 | 1:16 | 1:32 | 1:64 |
| 14 | 8/16 | 0 | 0 | 0 | 0 |
| 15 | | Soft shell, titer not determined | | | |
| 16 | 8/9 | 0 | 0 | 0 | 0 |
| 17 | 9/25 | +++ | +++ | ++ | ++ |
| 18 | 9/27 | +++ | +++ | +++ | + |
| 19 | 9/28 | +++ | +++ | +++ | +++ |

## CONCLUSIONS

1. The albumin of eggs from hens infected with *Bacterium pullorum* contained agglutinins specific for this antigen (exception in the case of fowl 7). Albumin of eggs from non-infected fowls rarely clumped the antigen above a trace in the 1:8 dilution.

2. Agglutination in the case of infected hens is most marked when a 1:8 dilution of egg albumin is used, though in some instances complete agglutination was observed in a 1:64 dilution.

3. There seems to be no correlation between serum and albumin titer.

4. One fowl (11) gave what appeared to be evidence of having acquired the ovarian infection as an adult as was shown by an increase in albumin and serum titer.

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# PRODUCTION OF MONOVALENT BOTULINUS ANTITOXIC SERUM TYPES A AND B

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Monovalent botulinus antitoxic horse serums types A and B have been prepared in this laboratory for prophylactic and therapeutic purposes. Although only a small number of animals, five horses, were used in this work, a record of the results of the methods employed is of interest and illustrates how differently horses may respond to immunization with these toxins.

Kempner (1), Forssman and Lundstrom (2), Graham and Brueckner, (3), and Dickson and Howitt (4) have reported on the production of fairly potent monovalent antitoxic goat serums. Kempner lost one goat out of three with chronic botulism. Dickson and Howitt found that the three goats used varied so in their reactions to the toxins that no stated method of immunization could be followed.

Leuchs (5) and Hart and Hayes, (6) produced monovalent horse serums, but methods of testing the toxins and serums vary so much that a comparison of the results is difficult.

Bengston (7) made tests on a limited number of polyvalent antitoxic serums according to the standard of the United States Hygienic Laboratory and concluded that it is apparently difficult to produce satisfactory polyvalent serums, antitoxin of one or the other type usually predominating. The serums tested contained from 2 to 40 units type A antitoxin, the type B antitoxin being much stronger. The highest titer obtained was 450 units.

## TOXIN PRODUCTION

The type A toxin used was prepared with strain A 16 received from Prof. M. J. Rosenau of Harvard University.

Two type B strains were used for producing toxins: one the Nevin strain, isolated by M. Nevin in this laboratory in 1914, the other a strain received from Professor Rosenau.

The regular van Ermengen broth was used with the following slight modification. In preparing the medium, the dextrose was omitted and the broth adjusted to pH 8.4 before sterilization, thus avoiding the increase in acidity which occurs during the sterilization of an alkaline dextrose medium. Sufficient 20 per cent dextrose solution to make a final 2 per cent was added at the time of inoculation.

Liter flasks of the broth inoculated with twenty-four to forty-eight hour broth cultures of the organisms were incubated at 33° to 35°C. for five weeks. The toxins were then preserved with 0.5 per cent phenol and filtered through pulp and finally through a filter candle to insure sterility. The potency of the toxins was determined by the subcutaneous method, according to the standard of the United States Hygienic Laboratory, the minimal fatal dose being the amount of toxin which, when injected subcutaneously, will kill a guinea-pig weighing 250 grams in about ninety-six hours.

### ANTITOXIN PRODUCTION

The monovalent antitoxic serums were produced by subcutaneous inoculation of horses with the homologous toxin. All but one of the five horses used had at times during the previous three or four years been under immunization with other toxins or bacterial antigens but had been allowed to rest for several months and were in good physical condition when immunization with botulinus toxins was begun. The titer of the serums was determined by testing against a standard toxin according to the method prescribed by the United States Hygienic Laboratory in which one-tenth unit of antitoxin protects against one test dose of toxin (about 100 M.F.D.'s). Bleedings of eight to nine liters were taken when the titer was sufficiently high. The serums were preserved with 0.3 per cent cresol and filtered, after three or four weeks, through a filter candle to insure sterility.

During the preparation of these serums, three methods of immunization varying slightly from one another were employed.

*Method I. Frequent injections of toxin with rapid increase in dosage:* In this method, used with type B toxin of one strain, subcutaneous injections of toxin were given at three-day intervals starting with an initial dose of $\frac{1}{20}$ M.F.D. of toxin and increasing the amount 40 to 50 per cent at each succeeding injection until the dose was 50 cc. Increases of 20 to 50 cc. of toxin were then made till the maximum dose of 500 cc. was reached. For the small doses, toxins of low potency M.F.D. 0.005 to 0.001 cc. were used. Later, toxins of higher potency were substituted. The first ten doses given by this method were $\frac{1}{20}$, $\frac{1}{5}$, 1, 2, 5, 10, 20, 50, 75, 100 M.F.D. of toxin. Three months after immunization was started the titer of the serum was about one unit of antitoxin per cubic centimeter. The maximum dose of toxin given thus far was 60,000 M.F.D. (60 cc. of toxin M.F.D. 0.001 cc.). At the end of five months when bleedings were taken, the titer of the serum was 150 units of antitoxin per cubic centimeter. At this time 500 cc. of toxin M.F.D. 0.0001 cc. were being given.

This same method was used in beginning the immunization of two horses with type B toxins of the other strain. The animals died with typical symptoms of botulism after the tenth and twelfth doses of toxin (100 to 140 M.F.D.). Another horse was then given repeated injections of 25 M.F.D. of toxin at three-day intervals to determine the cumulative effect of sublethal doses. Two days after the sixth dose, the horse developed typical symptoms of botulism and was killed.

Since apparently some horses would not respond readily to rapidly increasing doses of botulinus toxin, the method of immunization was modified.

*Method II. Slow increase of dosage with intervals of rest:* This method was used for immunizing one horse with type A toxin. A series of two doses of toxin was given on successive days followed by a rest of seven days. After the first two series of doses of $\frac{1}{20}$ and $\frac{1}{5}$ M.F.D. and 1 and 2 M.F.D. of toxin, the increase in the amount of toxin given in any one series was very gradual, not more than 5 to 10 M.F.D. Four months after immunization was started the serum had a slight protective action but the titer was still less than $\frac{1}{10}$ unit of antitoxin per cubic centimeter. At the end of five months the total amount of toxin given in the two doses of a series was 300 M.F.D. or 0.3 cc. of toxin M.F.D. 0.001 cc. The titer of the serum was between 1 and 10 units per cubic centimeter.

The doses of toxin were then increased more rapidly. At the end of seven months the titer of the serum was less than 50 units of antitoxin per cubic centimeter. The horse was in poor physical condition and was given a rest during which time he died but there was no evidence of botulism.

*Method III. Frequent injections of toxin with slow increase in dosage:* This method was tried with one horse immunized with type A toxin. Doses of toxin were given every third or fourth day as in the first method, but the amount of toxin given at each injection was increased very gradually. Thus, starting with an initial dose of $\frac{1}{20}$ M.F.D., the first ten doses of toxin were $\frac{1}{20}$, $\frac{1}{10}$, $\frac{1}{5}$, 1, 2, 4, 6, 8, 12 M.F.D. This gradual increase in the doses of toxin was continued for the first three months at which time the maximum dose given was 390 M.F.D. or 0.39 cc. of toxin M.F.D. 0.001 cc. The titer of the serum at this time was a little less than 1 unit of antitoxin per cubic centimeter. The doses of toxin were then increased rapidly, increases of 40 to 50 per cent being given as in method I, till the dose of toxin reached 50 cc., then increases of 20 to 50 cc. up to the maximum dose of 500 cc. The titer of the serum six months after immunization was started, when bleedings were taken, was 1400 units of antitoxin per cubic centimeter. Toxin of higher titer, M.F.D. 0.0002 cc., was substituted for the 0.001 cc. toxin but there was no marked increase in the titer of the serum up to the time immunization was discontinued two months later.

While the number of horses immunized by any of the above methods was so small that no definite conclusions can be drawn as to the relative value of any one method, the results suggest that the third method, of frequent injections of toxin with slow increase of dosage, may have certain advantages over the other two, since very potent serums can be produced by this method and there is less danger of animals succumbing during treatment.

## SUMMARY AND CONCLUSIONS

Potent monovalent botulinus antitoxic serums, types A and B, were produced by immunization of horses with homologous botulinus toxins. The maximum titer of 1400 units per cubic centimeter was obtained with type A toxin.

Not all horses respond to such rapid increases in the doses of botulinus toxin as were given in the first method of immuniza-

tion described. Two of the three horses immunized by this method succumbed after the tenth or twelfth dose with typical symptoms of botulism.

Antitoxin production with the one horse immunized by the second method (slow increase of dosage with intervals of rest) was slower than with the horses immunized by the other methods.

The small doses of toxin given at the beginning in the third method of immunization (frequent injections of toxin with slow increase in dosage) apparently stimulated antitoxin production as readily as the larger doses used at the beginning in the first method (frequent injections of toxin with rapid increase in dosage) and with less danger to the animal.

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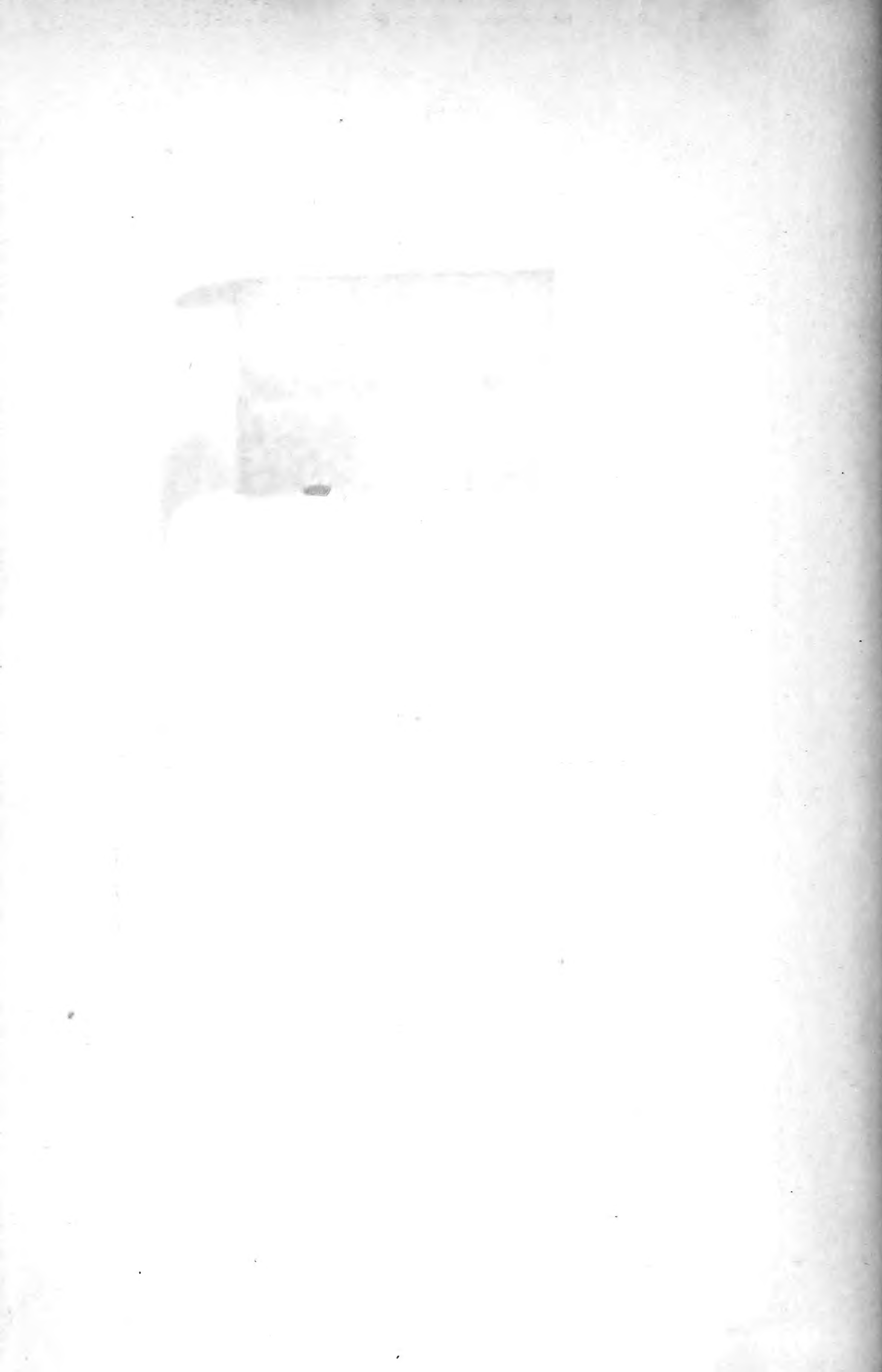

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