Vaccine therapy, its theory and practice / By R. W. Allen.

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VACCINE THERAPY

ITS THEORY AND PRACTICE

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BY

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LATE CLINICAL PATHOLOGIST TO THE MOUNT VERNON HOSPITAL FOR DISEASES OF THE CHEST; LATE PATHOLOGIST TO THE ROYAL EYE HOSPITAL; LATE GULL STUDENT OF PATHOLOGY, GUY'S HOSPITAL

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PREFACE TO THE THIRD EDITION

The author trusts that the present edition may be as favourably received as its predecessors ; it has been completely rewritten and brought up to date. The attempt has been made so to render available the experience of the past five years by the inclusion of illustrative cases as to enable the general practitioner to approach with confidence a case requiring therapeutical immunization. Accuracy in diagnosis, close clinical observation, and an open mind, are the prime essentials for success.

128, Harley Street, W.,
June, 1910.

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VACCINE THERAPY

CHAPTER I

OPSONINS: WHAT THEY ARE, THEIR NATURE AND SOURCE

Of the means whereby the body tissues are enabled to overcome bacterial invasion our knowledge is as yet far from perfect. The process is admittedly a very complex one. Various substances, to which the names 'agglutinins,' 'precipitins,' 'stimulins,' 'lysins,' and 'opsonins,' are given, are considered each to play a part in enabling the phagocytic cells to complete the destruction of the infecting bacteria. Metchnikoff holds that the principal part is played by the substances to which he has given the name 'stimulins.' The presence of these in the tissue fluids he has not yet succeeded in satisfactorily demonstrating, but considers their function to be that of acting upon the phagocytes so as to stimulate them to perform phagocytosis. While not denying the existence of opsonins, he assigns to them but a secondary part. Wright, on the other hand, has demonstrated beyond doubt the presence in the blood of substances which act upon the bacteria, and get them ready for the completion of their destruction by the phagocytes. To these bodies he has given the name of 'opsonins.' It would appear possible for phagocytosis to proceed without prior opsonization of the bacteria, unless it be argued —and this seems very plausible—that the phagocytic cells contain opsonins in their plasma fluid from which it is

hardly possible to free them. Be this as it may, it is beyond question that the presence of opsonin materially assists the processes of phagocytosis.

The method whereby the presence of opsonin in blood-serum is demonstrated is as follows : A little freshly-drawn blood is immediately received into eight or ten times its volume of 2 per cent. sodium citrate to prevent coagulation. The blood-cells are thrown down by rapidly centrifuging, and the supernatant liquid pipetted off. The cells are then thoroughly washed with a considerable bulk of a solution of 0·8 per cent. sodium chloride in distilled water, and again thrown down by means of the centrifuge, this process being repeated two or three times, so that finally the cells are washed practically free from all blood-plasma, and are left suspended in a very small volume of the normal saline solution, as uniform a mixture as possible being made. A twelve to eighteen hour old culture on agar of any organism—say *Staphylococcus albus*—is then taken, and a thick emulsion made with a solution of 0·1 per cent. sodium chloride in distilled water. Clumps are thrown down by means of the centrifuge, and the bacterial emulsion divided into two parts, *A* and *B*. *A* is set aside ; to *B* an equal volume of fresh blood-serum is added, and the two thoroughly mixed together and heated in an incubator at 37° C. for fifteen minutes. The bacteria are thrown down by means of the centrifuge, and as much liquid as possible pipetted off. The precipitated bacteria are well washed with 0·1 per cent. solution of sodium chloride in distilled water, and again thrown down, this process being repeated several times. Finally, an emulsion of the bacteria is made in the salt solution exactly like *A*, and the numbers present respectively in emulsions *A* and *B* counted, the thicker emulsion being diluted to exactly the same strength as the weaker. We thus

have a suspension of blood-cells of which unit volumes contain the same number of polymorphonuclear white cells—*i.e.*, of phagocytes—and two emulsions of the same strength of a given organism in 0·1 per cent. salt solution, differing only in the fact that the bacteria in one (*B*) have been acted upon by blood-serum at 37° C. for fifteen minutes. If this has had no action upon the organisms, identical results should be obtained by the following procedure: Equal volumes of the blood-cells and the bacterial emulsion *A* are thoroughly mixed together in a capillary pipette and incubated at 37° C. for fifteen minutes, the same being done with the substitution of emulsion *B* for *A*. Films are spread, stained by Leishman's method, and observed under a $\frac{1}{12}$-inch oil-immersion lens. The number of bacteria engorged by 100 polymorphonuclear leucocytes is then counted upon each film. An experiment performed in this way gave the following result:

Bacterial Emulsion employed.	Number of Cocci in 100 Polymorphonuclear Leucocytes.
A	10
B	500

It is thus obvious that some change is produced in the bacteria by the action of the blood-serum whereby phagocytosis is expedited. To the substance by which this change is brought about Wright gave the name 'opsonin.'

The Nature and Constitution of Opsonins.

Until they have been isolated and obtained in a state of purity, it is obvious that the exact constitution of opsonins cannot be determined.

Certain observations render the view probable that they are of a proteid nature. Thus, Yorke[1] filtered normal

[1] *Biochemical Journal*, vol. ii., June, 1907, p. 357.

serum through a sterile Chamberland candle under very high pressure, and found that the opsonin passed readily through for the first few minutes, but that after that only traces permeated the candle-wall, owing to the pores being filled up by the proteids of the serum. The residue in the filter, beside containing comparatively unaltered serum, consisted also of a gelatinous substance, adherent to the sides of the candle and of high opsonic power. It would thus appear that opsonins will not pass through a Chamberland candle the pores of which have been blocked up with gelatine or proteid substance. They would therefore appear to be of a 'colloidal' nature. Lamar and Bispham[1] showed also that they were not dialysable, and that they are carried down with the euglobin when serum is half saturated with ammonium sulphate.

In certain respects they bear some resemblance to the ferments. Thus, serum can be diluted to a considerable extent without marked lessening of its opsonic power. Noguchi[2] has also shown that they are not destroyed by drying the serum at 23° C., and that in this desiccated state they retain their activity after two years, and are, moreover, comparatively resistant to heat. Exposure to a temperature of 120° C. but slightly impairs their power, which is not altogether destroyed by a temperature of 150° C.

Like ferments, opsonins are also very sensitive to slight alterations in the acidity or alkalinity of the medium in which they are dissolved, displaying the greatest activity in a solution of neutral reaction. As regards their biological constitution diverse views are held. It would, however, appear that the opsonins present in normal serum and in that of an infected or immunized animal, which is known as an 'immune' serum, are not quite the

[1] *Journal of Experimental Medicine*, December, 1906.
[2] *Ibid.*, vol. ix., No. 4, p. 455.

same thing, and the elucidation of their nature has been much hindered by the failure, especially of the earlier investigators, to recognize this possibility.

The following experiment of Yorke and Smith[1] upon normal serum corrected earlier observations by Bulloch and Western, and has been amply confirmed by other investigators. A strong emulsion was made of anthrax bacilli in 0·9 per cent. NaCl solution, and killed by heating at 100° C. for thirty minutes. The bacilli were thrown down by centrifuge, washed thrice with 0·9 per cent. NaCl solution, the washed dead bacteria made up into a strong emulsion, and added to two equal portions of 'normal' serum, *A* and *B*. *A* was incubated at 37° C. for thirty minutes, *B* for sixty minutes.

The bacteria were then thrown down, and the supernatant sera tested for opsonin with staphylococcus and anthrax. The control sera were diluted to an equal extent with 0·9 per cent. NaCl solution and incubated for similar times with the like organismal emulsions. The figures obtained from the films prepared therefrom were as follows:

TABLE I.

	Number of Bacteria phagocytosed by 50 Leucocytes.	Index.
A.—I. Anthrax:		
Control serum	40	1·00
Treated serum	8	0·20
II. Staphylococcus:		
Control serum	341	1·00
Treated serum	87	0·25
B.—I. Anthrax:		
Control serum	48	1·00
Treated serum	2	0·04
II. Staphylococcus:		
Control serum	395	1·00
Treated serum	70	0·17

[1] *Biochemical Journal*, vol. ii., December 19, 1906.

Other experiments gave similar results, which have been confirmed by Simon, Potter, and others. It therefore appears that the incubation of a large number of any organism with serum will not only greatly reduce the contained opsonin for that given bacterium, but those for other organisms as well. In other words, much the greater proportion of the opsonin present in 'normal' serum is 'non-specific.' As to how much of the remaining proportion of the opsonin is 'specific,' exact observations are lacking.

Numerous experiments have been performed which demonstrate a difference in the behaviour of the opsonins of 'normal' and 'immune' sera. Thus, Bulloch and Western repeatedly tested the serum of human beings against both staphylococcus and the tubercle bacillus. Injections of tuberculin were then given, and found to produce a rise in the tuberculo-opsonin, while not affecting the staphylococcic opsonin. Injections of killed staphylococci had the reverse effect.

The fact, too, that infected patients are found to have either a high or a low index towards that particular organism, and a normal index towards all others, points to an alteration in the opsonin produced by the infection. That this has resulted in the production of a 'specific' opsonin is rendered probable by numerous observations, of which the following may be given as an example :

If two sera, *A* and *B*, be taken, *A* being a 'normal' serum, *B* that of a person infected by the tubercle bacillus —*i.e.*, an 'immune serum'—and each of these sera be divided into two portions, the one of which is heated at 60° C. for half an hour, the other not, and the opsonizing power of these four specimens of serum towards the tubercle bacillus be estimated, a result like that set out in the following table will be obtained :

	Normal Serum.		Immune Serum.	
	Unheated Portion.	Heated Portion.	Unheated Portion.	Heated Portion.
Bacteria in 100 leucocytes	300	15	200	80

Whereby it is seen that the effect of heating the serum has been very different in the cases of the 'normal' and 'immune' sera respectively. In other words, the amount of 'thermostable' opsonin for the tubercle bacillus is much greater in the 'immune' than in the 'normal' serum.

A comparison of the opsonizing powers towards staphylococcus in the case of these sera would reveal no difference in behaviour.

It is therefore probable that, as a result of the tubercular infection, the amount of 'specific' thermostable opsonin has been increased.

To sum up, while it must be admitted that the demonstration of 'specificity' of opsonins even in 'immune' sera is not complete, it is yet highly probable that in the blood-serum of a perfectly healthy individual there is a minimal amount of opsonin specific against the various pathogenic bacteria, while much the greater proportion is non-specific. The suggestion has been made that opsonin does not so exist in blood or tissue plasma as such, but as 'opsinogen,' needing contact with bacteria or other substances for the formation of 'opsonin,' just as 'fibrin' exists in the blood as fibrinogen, needing calcium salts for its conversion into fibrin. In an individual infected by a given bacterium the amount of opsonin specific against that bacterium undergoes considerable variation from the normal, and is probably increased in every case, not necessarily beyond the amount of 'specific'

opsonin which the healthy individual is capable of elaborating, but beyond the amount which he actually does normally elaborate. Assuming that among the other protective mechanisms of the body a quantity '*A*' of 'specific' opsonin is necessary to enable a given individual to overcome an infection by a certain bacterium, his capacity for elaborating this specific opsonin '*A*' may be in excess, exactly adequate, or in default. In the first and second instance the infection will be overcome in a time varying, *inter alia*, with the amount of specific opsonin elaborated. In the last instance it will not be overcome until such time as his capacity is raised to the necessary point, or unless the other defensive mechanisms of the body suffice.

As regards the structure of opsonins, the following possible views all have their advocates—

1. That opsonins are identical with certain other immune bodies.

(1) Amboceptors (Savtchenko).

(2) Complements (Levaditi, Inmann).

2. That opsonins are not identical with these other bodies, but—

(1) Have a simple structure like toxins, agglutinins, precipitins, amboceptors, complements ; or—

(2) Have a double structure, like cytotoxins and hæmolysins, needing the co-operation of a thermostable, amboceptor-like body, and a thermolabile, complement-like body (Muir and Martin, Dean, Cowie, and Chapin, etc.).

3. That opsonins are unlike any other antibody, and form a class by themselves (as originally upheld by Wright and Douglas, Bulloch and Atkin, Keith, Hektoen, Neufeld, and others).

To enter into a full discussion of all those possibilities is quite outside the scope of this book ; the theory ren-

dered most probable by the weight of present evidence is the second of these, which may be enunciated as follows: Opsonic action is the effect of two bodies acting together —one, thermostable and of amboceptor-like nature, is the essential substance; alone, it is perhaps capable of opsonizing, but its activity is greatly increased by the presence of a thermolabile, complement-like body. The amboceptor-like constituent is present only in very small quantity in normal serum; hence the apparent thermolability of the opsonin in normal serum, whereas in an 'immune' serum the amboceptor plays the predominant part; and though heating results in a loss of activity, this is only partial. In the case of both normal and immune serum this loss is due to the destruction of the complement-like constituent. Considerable support is lent to this view by recent experiments, and notably those of Cowie and Chapin,[1] whereby they showed—

1. That heated normal serum may be reactivated by the addition of small amounts of fresh normal serum, a phagocytosis resulting which is greater than the sum of the phagocytoses of the sera taken separately. A similar result is obtained with heated 'immune' serum—*i.e.*, the addition of complement in the fresh serum assists the amboceptors of the heated serum.

2. That just as ordinary amboceptors can effect combinations at the freezing-point, while complements cannot, so with opsonins. Thus, normal serum may have its opsonic power for staphylococcus removed by addition of sufficient staphylococci, the mixture being maintained at a temperature near 0° C. throughout.

That this has resulted in the binding of the amboceptor-like constituent while the complement remains free is

[1] *Journal of Medical Research*, October, 1907, and February, 1908, p. 57 and p. 95.

shown by the fact that serum so treated—the bacteria with bound amboceptor having been removed by centrifuge—may still have the power to reactivate a heated serum.

3. Bacteria so treated—*i.e.*, bound to amboceptor—if thoroughly washed with cold salt solution to remove adherent complement, are not much more susceptible to phagocytosis by blood-cells, washed free from complement, than they were before ; but the addition to the mixture of complement, either in a little dilute normal serum or in serum inactivated by contact with bacteria in the cold, results in a phagocytosis greatly above the normal.

4. Staphylococci so treated—*i.e.*, bound with amboceptor—are much more easily opsonized by dilute normal serum, or by serum which has been inactivated by contact with staphylococci in the cold, than are the same bacteria not so treated.

5. That a heated serum loses its power to be reactivated if previously treated with a sufficient number of staphylococci—*i.e.*, if it has its amboceptors thus removed.

Further support is lent to this view by the fact, pointed out by Muir and Martin (1906 and 1907), that in the case of the thermolabile constituent of normal serum various substances which absorb complement also absorb opsonin—viz., erythrocytes, bacilli, and serum—when combined with their corresponding antibodies—viz., hæmolytic and bacteriolytic amboceptors and precipitins respectively—whereas these substances have little or no effect upon the thermostable constituent of an immune serum.

The experiments of Meakins,[1] however, tend to show that in certain instances, at all events, opsonin and amboceptor are not identical, for in the production of active immunity the two may not be increased to anything like the same extent.

[1] *Journal of Experimental Medicine*, January, 1909, p. 100.

Anti-Opsonins: Specific and Non-Specific.

Hektoen and Ruediger[1] have shown that many substances, such as calcium and barium chlorides, sodium bicarbonate, lactic acid, and alcohol, have the power of inducing a marked general fall in the opsonic power of the blood-serum. The addition of any alkali has the same effect as has that of any acid after the alkaline reaction of the serum has been reduced past the point of neutrality. These substances would therefore appear to be non-specific anti-opsonins. Upon the other hand, if a healthy man be injected with antitetanic serum, a specific rise in the tetano-opsonic index first occurs. This is followed, however, by a general fall. Thus, the staphylococcal, tuberculo-, and tetano-opsonic indices all fall below normal (Yorke and Smith).[2] A similar, though less marked, general depression is observed after injection of antistreptococcal or antidiphtheritic serum. R. Bradshaw[3] has recorded the following observations upon the effect of injections of antidiphtheritic serum upon the tuberculo-opsonic index.

TABLE II.

No. of Case.	Interval since Anti-diphtheritic Serum given.	Index.
1	2 days	1·30
1	5 ,,	0·64
2	12 ,,	1·02
2	25 ,,	0·35
3	27 ,,	0·89
3	41 ,,	0·72
4	25 ,,	0·64
5	26 ,,	0·72
6	26 ,,	0·62
7	27 ,,	0·77
8	28 ,,	0·69
9	3 months	0·47

[1] *Journal of American Medical Association*, May, 1906.

[2] *Biochemical Journal*, 1906, p. 341.

[3] *Lancet*, May 19, 1906, p. 1387.

Banks[1] found a slight but definite rise in the tuberculo-opsonic index within a few days of the injection of anti-diphtheritic serum, which became converted into a slightly lowered index within a few weeks. The changes were, however, so small as to lie within the limits of experimental error, and, granting that they do represent the actual result, the effect of the serum as serum needs to be taken into consideration.

Other observers do not agree with the observations either of Bradshaw or Banks, but find that an initial fall of slight duration is followed by a pronounced subsequent rise. So much is this the case that marked improvement is claimed to have been observed in tuberculous subjects to whom antidiphtheritic serum has been administered.

The experiments of Hektoen and Ruediger (*supra*) confirm the conclusion that the injection of these sera results in the formation of specific anti-opsonins.

Site of Formation of Opsonins.

That opsonin is not formed in the blood is practically certain. The amount of opsonin present in the blood bears no definite relation soever to leucocytosis, nor is it affected by disease of the blood-forming organs. Evidence is forthcoming that it is a product of muscular or subcutaneous activity. Allen has shown both in man and animals that if limbs be thoroughly perfused with normal salt solution to remove all blood, and the muscles cooled and minced and their plasma extracted in the usual manner, that the index of this plasma, despite slight dilution with the saline solution used in the perfusion, is markedly higher than that of the blood-serum towards

[1] *Lancet*, June 26, 1909, p. 1825.

various organisms. In the instance of an amputated leg the index of the muscle-plasma compared with that of the patient's serum was 1·4 towards the bacillus of Friedländer, the tubercle bacillus, and *Staphylococcus aureus*. In another case it was found to be 1·3. From which it may be concluded that actual formation of opsonin occurs in the muscle or subcutaneous tissues, and passes thence into the blood. This local formation explains the experience of Wright[1] that a certain case of tubercular ulceration which had previously defied treatment did well when the tuberculin was injected in a concentric manner around the area of ulceration.

To the bone-marrow and spleen the rôle of opsonin formation has also been ascribed upon somewhat slender evidence.

Fate of Opsonins in the Organism.

As regards this question but little is known. It, however, appears that all exudates and secretions contain certain amounts of opsonin. Miller found that it is contained in appreciable amount in the sweat, and to a larger degree in the urine, and that this excreted opsonin is increased during a negative phase consequent upon the injection of a bacterial vaccine. Milk also contains opsonin, perhaps to the extent of a quarter or a fifth of that of the blood, so that the question as to whether the opsonin of the mother's milk can be absorbed through the alimentary tract of the infant attains considerable importance. Wells,[2] from a study of the indices of breast-fed and artificially-fed infants, has concluded that no advantages in this respect are possessed by the former over the latter.

[1] *Lancet*, August 24, 1907, p. 494.

[2] *Practitioner*, May, 1908, p. 635.

CHAPTER II

DETERMINATION OF THE OPSONIC CONTENT OF THE BLOOD

Definition of the Opsonic Index.

The opsonic index may be defined as the ratio :

$$\frac{\text{Opsonic content of unit volume of the patient's blood-serum}}{\text{A normal person's}}$$

This is determined according to a method first introduced by Leishman for the estimation of the phagocytic power of blood, as modified by Sir Almroth Wright.

The following materials and apparatus are required :

1. A sufficient quantity of the patient's blood-serum and of that of the normal person.

2. Blood-cells which have been thoroughly freed from the plasma in which they normally float.

3. An emulsion of the bacterium towards which the opsonic index of the patient is to be determined.

4. Glass-tubing $\frac{3}{16}$ inch and $\frac{5}{16}$ inch in external diameter —the smaller for collection of the blood samples, the larger for the opsonic determinations. The former are to be cut into lengths of about 3 inches, and drawn out into capillary threads at each end, which are then cut off short. The latter are to be drawn out at one end only into fine capillary threads about 6 inches long and as far as possible of uniform bore.

5. Strong rubber teats, file, grease pencil.

6. Centrifuge with hæmatocrite attachment, and glass tubes to fit the same.

7. Watch-glasses and platinum loop.

8. The following solutions in sterile distilled water, carefully freed from dust and hairs, not by filtering, but by centrifugalization :

(*a*) 1·5 per cent. to 2 per cent. neutral sodium citrate.

(*b*) 0·8 per cent. sodium chloride.

(*c*) 0·1 per cent. sodium chloride.

9. Glass slides thoroughly grease-free.

10. Incubator (Hearson's biological), maintained at 37° C.

11. Methylic alcohol for fixing.

12. Appropriate staining solutions—viz., for all organisms except tubercle, Leishman's stain ; for tubercle, carbol fuchsin, 10 per cent. sulphuric acid, absolute alcohol, and toluedene blue.

13. Porcelain jar with metal cover for holding slides during fixing and staining.

14. Microscope with $\frac{1}{12}$-inch oil-immersion lens and mechanical stage ; cedar-wood oil.

The following procedure is then to be adopted :

1. *Collection of Blood for Serum.*—This is done by cleansing the finger-tip or lobe of the ear with warm soap and water or 2 per cent. lysol solution, drying, and rubbing well with a small piece of lint saturated with ether. When the latter has evaporated, a prick is made with a needle. This is best done decisively, for patients prefer one effective puncture to several ineffective ones. As a rule they prefer the finger-tip to be utilized, but should the epidermis be obviously thick at the root of

the nail, it is better to employ the lobe of the ear. The blood must flow spontaneously, or but very slight pressure be employed, and the first drop wiped away, for, as has been shown, the opsonic content of the plasma of muscle and the subcutaneous tissues is considerably higher than that of the blood. On approximating one of the capillary ends of the tube to the blood, the latter will flow spontaneously into it. Three or four drops of blood will suffice. The tube must now be sealed off, and here a word of caution is necessary; opsonins are readily destroyed by heating to 60° C.; the blood must, therefore, not be heated. All risk of this is avoided by gently warming the end of the tube away from the blood, and then sealing off this end. Lay the tube down flat, and allow it to cool. In doing so the blood is sucked back from the unsealed capillary end by the vacuum produced by the contraction of the contained air as it cools. When this has occurred, that end also may be sealed off in the tip of the flame. These precautions are far from unnecessary, for I have seen many samples of blood quite spoilt in the collecting. The control blood should be taken at the same time as that of the patient.

2. *Preparation of the Blood-Cells.*—The blood for this purpose should not be collected from a sufferer from disease of the lymphatic system, or from an individual whose red blood-cells are capable of agglutination either by their own serum or by that from any other source. As Fleming[1] has pointed out, this is particularly liable to occur in the case of infected individuals, and the effect of agglutination of the red cells in an opsonic mixture is to give an unduly high phagocytic count.

Aseptic precautions should be taken to prevent con-

[1] *Practitioner*, May, 1908, p. 607.

tamination with organisms which will grow rapidly in such a favourable medium, and prove troublesome, perhaps, when the time comes for counting the slides. The collection is done in one of the $\frac{5}{16}$-inch glass pipettes, to which a strong rubber teat has been fixed. A little of the sodium citrate solution is first sucked up to prevent coagulation, then the blood, which is at once transferred to a tube containing more of the sodium citrate solution. Blood may be added to the citrate in the proportion of 1 to 5.

The citrate, by precipitating the calcium salts of the blood, effectually prevents coagulation. The citrated blood is now transferred to the centrifuge tubes and thoroughly centrifugalized. A very considerable speed —10,000 revolutions per minute—may be advantageously employed ; the corpuscles will be thrown down quickly and yet escape damage. It is to be remembered that the white cells are lighter than the red, and will therefore be thrown down last. It is well to continue the operation till a distinct white layer is seen lying upon the layer of reds, for efficient centrifugalization means numerous white cells, and so greater facilities in counting. The clear supernatant citrate solution is pipetted off, care being taken not to disturb the white layer. Some of the 0·8 per cent. sodium chloride solution is now added to the cells, and these thoroughly mixed up with it and again thrown down. Concentration of the white cells may be effected by removing the upper layer of cells from one tube, adding these to the second tube, the lower layer in the first being then thrown away. The washing with normal saline solution is repeated once or twice. As much of the liquid as possible is finally removed ; the cells, thoroughly mingled with what is left, are then ready

for use. A little plug of cotton-wool will prevent access of organisms from the air.

3. *Preparation of the Bacterial Emulsion.*—For this purpose, whenever possible, a culture of the patient's own organism should be employed, for, as Lohlein[1] has pointed out, the individual qualities of a given strain are of marked influence on the resulting phagocytosis. As a rule, the more virulent the bacterium, the greater is its resistance to phagocytosis. Young organisms stain better and more uniformly than old. It is, therefore, better to employ as recent a culture as possible, especially in the case of such organisms as that of Morax-Axenfeld, which begin to involute even before eighteen hours. A twelve- to sixteen-hour-old culture on an appropriate medium—such as agar for staphylococci, streptococci, coli, etc. ; blood-agar for gonococci ; nutrose ascitic agar for *Bacillus Morax-Axenfeld* or *Micrococcus catarrhalis*—is, therefore, to be employed. If fresh blood-serum or ascitic fluid enter, however, into the composition of the medium, it should be heated at 60° C. for half an hour before addition to the agar ; otherwise the organisms cultured thereupon will be subjected to opsonization during growth. If the growth be a very copious one, it is best to take a loopful of the culture on a platinum wire, and carefully emulsify it in a watch-glass with a little of the 0·1 per cent. NaCl solution. If the growth be scanty, then it is best to pour a few drops of the solution into the culture-tube and emulsify it *in situ*. The turbid emulsion thus produced contains many clumps, which are to be thrown down by means of the centrifuge. A minute or two will usually suffice at a high speed, but experience alone will teach just how long it should be

[1] *Ann. de l'Instit. Pasteur*, 1906.

continued. In any case, it must be efficient, for nothing is more annoying than to find clumps in the films when everything has been completed, for if accuracy be desired the whole process must then be repeated. Experience, again, alone will teach whether the emulsion requires further dilution. The opacity of an emulsion, say, of gonococcus must be much greater than that of emulsions of staphylococci or Friedländer's bacillus in order to give the same count in the normals. A strength which will give a count of about 250 to 350 bacteria in 100 white cells of the normal should be aimed at. In the instance of the tubercle bacillus an emulsion once made and found satisfactory may be preserved sealed up in capillary tubes for practically any length of time, especially if the bacteria have been killed by heating to 70° C. for one hour. When wanted, all that is necessary is thoroughly to shake up the emulsion and give it a few sharp turns in the centrifuge to throw down any clumps which may be present.

These preliminaries over, we now take as many of the fine long-drawn capillary pipettes as there are sera to be investigated. They should be chosen of as equal bore as possible. It is advisable for them to have been sealed off at the fine extremity, plugged with cotton-wool at the other, and dry sterilized. The fine ends are cut off square by means of a file scratch, and marks made with a grease pencil about 1 centimetre from the ends. The content as far as this mark is the unit volume in each case. To the plugged ends are fitted the strong rubber teats, and each pipette is marked with a number corresponding to a serum. The rubber teat is now held between thumb and forefinger and gently compressed, the capillary end inserted into the well-mixed blood-cells, and the unit

volume drawn up by slightly relaxing the pressure on the teat. Next a tiny bubble of air is allowed to enter, a second and third volume of blood-cells being drawn up in similar fashion, each separated from the next by a bubble of air. A volume of the bacillary emulsion is now drawn in with especial accuracy, then a bubble of air ; finally, 2 volumes of the serum, which must be taken up free from admixture with red cells, as these tend to produce an unduly low phagocytosis. We thus have in order in the pipette 3 volumes of blood-cells, 1 volume of emulsion, 2 volumes of serum, each volume being separated from the adjoining by means of a bubble of air. This is the procedure usually followed, but if the emulsion be suspected to be too thin, then 2 volumes of blood-cells, 1 volume of emulsion, and 1 of serum may be employed, or the original 1, 1, 1 of Wright. The order—cells, emulsion, serum—should, however, always be followed, for in this way contamination of the cells by the bacterial emulsion, or introduction of opsonin from the serum into the emulsion, is avoided. By gentle pressure on the teat the several volumes are expressed on to a clean glass slide, and thoroughly mixed by alternately sucking the mixture into the pipette and squeezing it out again upon the glass slide. Only by thorough mixing can a satisfactory count be ultimately obtained. The mixture is finally withdrawn as completely as possible some little distance into the pipette, and the extremity sealed off in the flame.

This operation is repeated with each serum. The several pipettes, carefully labelled, are placed in the incubator at 37° C. for fifteen minutes. By means of a file-scratch the ends are then cut off, the content of each blown out on to a clean glass slide, and very carefully

mixed. Half the drop is transferred to a second slide, and two blood-films prepared by the slide method—*i.e.*, by drawing the extremity of one slide held at an acute angle over the surface of the other upon which the drop of blood has been placed.

Mention may here be made of two points of some importance: Firstly, the thickness of the blood-film depends partly upon the pressure employed in the spreading, and to a greater extent upon the inclination of the moving slide to the stationary one. The more vertical the former is held the thinner the film, and, conversely, the more acute the angle the thicker the film. Now, the ideal film is one in which the corpuscles do not lie one upon the other, but are even separated by distinct intervals, for in such an one the white blood-cells flatten out, and consequently are of larger size. The contained bacteria are, therefore, much more easily distinguished after staining, and counting is consequently facilitated. Films containing tubercle bacilli may, however, be spread rather thicker than in the case of other organisms, for the staining methods are more drastic, the organisms show up more clearly, and the red cells are practically invisible.

To obtain the best films firm pressure should, therefore, be employed, and the slides should be held at an angle of 60 degrees to one another.

Secondly, owing to their greater viscosity, the white cells tend not only to be drawn towards the end of the film, but also to run to the edges. These facts may be turned to practical advantage if the precaution be taken not to place too large a quantity of blood upon the slide. Instead of using a slide of ordinary breadth for spreading, one may be bisected longitudinally by means of a glazier's

diamond, and this half-slide employed. If the drop of blood be then placed at the mid-point of the breadth of the slide, but near one extremity, and the half-slide used as a spreader, a film is obtained with two edges lying some little distance from the margins of the slide, and along these edges the white cells will be found collected. Next, by moving the spreader in a series of little jerks instead of with a uniform motion, a number of little valleys, as it were, are made in the film, in which the white cells collect just as they do along the edges.

Attention given to these trifling details is well repaid by the additional ease with which the slides are counted.

The spread films are allowed to dry in the air; one of each is reserved in case of accident; the others are treated as follows: If containing tubercle bacilli, they are fixed for fifteen minutes in methylic alcohol, or for one hour in a mixture of equal volumes of ethyl alcohol and ether, stained by the Ziehl-Nielsen method, and counterstained with toluedene blue. Five minutes' application of the latter stain, followed by thorough washing under the tap, will show up the bodies of the white cells most effectually. For any other organism than the tubercle bacillus the films are best stained according to Leishman's method.

Next, with $\frac{1}{12}$-inch oil-immersion lens and a mechanical stage the numbers of bacteria contained in each consecutive five polymorphonuclear leucocytes are noted till 100 cells have been counted. No estimation can be considered satisfactory unless the numbers of bacteria found in each five cells approximate to each other. The following points may here be noted, and too much stress cannot possibly be laid upon their importance if accuracy be

desired in the estimation : Firstly, the advisability of counting as many cells and their bacterial contents as possible. Reliance is commonly placed upon a count of fifty cells. I would maintain that no amount of care at every stage will insure an accurate result with such a count ; 100 cells is the minimum number that should be observed. Secondly, the occurrence of bacterial clumps of any size in a film, especially if these lie upon any of the cells, should damn such a film beyond redemption. There is nothing for it but to repeat that experiment with that serum, and, of course, with a fresh normal. Thirdly, the occurrence of clumps of leucocytes, especially if these be held together by threads of fibrin, should render the experiment null and void. Once more repetition is more than advisable. Of course, both these last difficulties should not occur ; they are, as a rule, the result simply of lack of care in preparing the blood-cells and the bacterial emulsion.

The determination of the index is now completed as follows : The normal serum is taken as having an opsonic index of unity. The number of bacteria found in 100 cells of each of the patient's slides divided by the number in 100 cells of the normal slide gives their respective indices. To recapitulate, then, the points of importance, by observance of which accuracy can alone be secured and much time and trouble saved :

1. The solutions used for the preparation of the blood-cells must be quite free from hairs and filaments, for these inevitably entangle the white cells and lead to clumps in the films.

2. The blood must be received into sufficient citrate solution to insure complete prevention of clotting, and the cells, when washing is complete, must be thoroughly

mixed to insure equal numbers of leucocytes in equal volumes.

3. The bacterial emulsions must be thoroughly centrifugalized to free them from all clumps, and growths of not more than eighteen hours should be employed for their preparation. The strength should be such that 250 to 350 bacteria are found in 100 cells of the normal.

4. The several volumes must be thoroughly mixed, both before and after incubation, to secure uniformity of count in each series of five cells.

5. The films must be spread thinly to insure the polymorphs being as large as possible.

6. Staining must be satisfactory, and the cell-body shown up. If this prove not so, the reserve slide must be stained.

7. At least 100 cells in each film should be counted, and to obtain this number it should be necessary to search a considerable area, including both ends as well as the centre of the film.

8. If at the first attempt an unsatisfactory result is obtained, whether from clumps of cells or bacteria, or from too few white cells being present in the films, perseverance in counting the imperfect films is to be deprecated. Time and temper will alike be saved by repeating the whole estimation.

Brief reference may here be made to recent attempts at shortening the technique in determinations of tuberculo-opsonic indices by the employment of emulsions of killed organisms which have been already stained with carbol fuchsin. Although one or two observers[1] have reported favourable results, the more general experience

[1] Campbell, *British Medical Journal*, April 13, 1907, p. 866.

is that accuracy cannot be thereby secured. The chief difficulty appears to be in the preparation of a satisfactory emulsion free from clumps. Staining, whether by weak and cold or hot and strong fuchsin solutions, seems to affect the organisms in such a way that centrifugalization, which throws down the clumps, also suffices to throw down the single bacilli.

The Question of the Accuracy of this Method of Estimating the Opsonic Content of the Blood.

It must be admitted that the reliability of estimations of the opsonic content of the blood, conducted according to the above technique, or slight modifications of it, has been rudely assailed during the past two years—among others by Simon, Lamar, and Bispham,[1] and by Walker[2] in America, and by FitzGerald, Whiteman, and Strangeways[3] in England.

In these un-Socratic days the honesty of all, both of those upholding and of those opposing any given procedure, is to be assumed. Against their experiments and results, then, are to be placed those of Wright and his co-workers, Bulloch, White,[4] the author, and many others. Upon the one hand are those who obtain unreliable results ; upon the other those who truly believe, and, as far as figures can substantiate a belief, find support in their figures for the belief, that in their hands the method affords reliable results.

[1] *Journal of Experimental Medicine*, August, 1906, p. 651 ; *ibid.*, September, 1907, p. 485.

[2] *Journal of Medical Research*, July, 1907, p. 521.

[3] *Bulletin of Committee for Study of Special Diseases*, Cambridge vol. i., No. 8.

[4] *Practitioner*, May, 1908, p. 639.

It thus follows that there are two classes of observers : (1) those who can estimate an index accurately by these methods ; and (2) those who cannot estimate an index accurately by these methods—just as there are surgeons who can perform perfectly the delicate operations advocated by Mayo Robson and by Arbuthnot Lane, and others who cannot. Upon the one hand, the possibility of the proper performance of these operations cannot be impugned by any multiplicity of ill-results in unskilled hands ; upon the other hand, the possibility of the accurate estimation by this method of the opsonic content of the blood is not disproved in the slightest by any number of inaccurate estimations in unskilled hands.

Let me not be misunderstood. I make no claim that the accuracy of the method is comparable with that, say, of the determinations of the various physical coefficients. There are pitfalls innumerable for the unwary, and even the most skilled experimenter will every now and again obtain a wrong result ; but here is an important point : he will know that he is obtaining an unreliable result, and will either repeat the whole estimation or count a much larger number of cells than usual, and so minimize the error. Fleming[1] appositely remarks : ' In this connection it should be pointed out that it is a great mistake to have any arbitrary number of leucocytes which one counts, neither counting more nor less, whatever the conditions may be '—a remark with which I most completely agree ; and again : ' Intelligence must be brought to bear on the subject in hand.'

Despite this, it cannot but be admitted that even in the hands of the most skilled there is, as Greenwood[2] points out, an error inherent in any such method which

[1] *Practitioner*, May, 1908, p. 627. [2] *Ibid.*, p. 641.

is quite inevitable—an error, however, which only rarely exceeds 10 per cent., and is usually about 5 to 6 per cent., and is of little or no practical importance.

The value of the index thus determined as a guide in immunization is discussed subsequently (see p. 80).

Other Methods of Estimating the Opsonic Content of the Blood.

Inasmuch, then, as the above method of determining the opsonic index must be admitted to be a very delicate and somewhat laborious and difficult matter, and one, moreover, which is unsuited to a certain proportion of workers, any other method which is less laborious, less difficult, and suitable for all, can only be regarded as in the highest degree desirable.

Several attempts have been made in this direction. Thus, Simon (*v.s.*) would substitute an index obtained by diluting the blood in varying proportion (ten to thirty times), and after incubating with a bacterial emulsion of considerable strength, comparing the percentage of phagocyting leucocytes in the specimen of blood under investigation with the figure obtained after a similar procedure with a specimen of normal blood. This he calls the 'percentage index,' and finds it sometimes to agree well with the opsonic index, sometimes to differ considerably from it, in which event he prefers to follow the guidance of his percentage index. As to the accuracy of the method I can offer no opinion, but it is admittedly even longer than the method it seeks to displace, and to my mind has this very grave objection : the dilution of the serum. It is perfectly true that if the opsonizing power of the serum of an infected individual be com

pared with that of the serum of a healthy individual, marked differences are revealed according as the undiluted sera or the sera in various degrees of dilution are compared. As dilution proceeds, it will sometimes be found that the opsonizing power of the immune serum rapidly falls off in comparison with that of the normal serum. Thus, an index of 1·4 may be obtained for the undiluted immune serum, and an index of only 0·8 for the same serum in a dilution of 1 in 20. Certain observers—as Simon and Walker (*v.s.*), who estimate the opsonic index by means of diluted sera—consider examination of such to afford the better idea of the immunizing power of the blood. I would maintain that they are conducting an investigation—the results of which are doubtless of value—under conditions which do not in the least obtain in any pathological condition in the human organism. If possible, what one desires to ascertain is the opsonizing power, not even of the blood-serum, but of the blood-plasma, in the condition in which it actually is in the human organism. Moreover, not even in a suppurating focus nor in an exudate of pus do the bacteria ever present any such ratio to the phagocytes as they employ in their phagocyting mixtures. In order to learn the immunizing response of the body, say, in a case of streptococcal septicæmia, where, perhaps, ten streptococci can be isolated from 5 c.c. of blood, they would present an emulsion containing at least 5,000 million organisms per c.c. to a mixture of blood-cells containing, perhaps, 5,000 to 10,000 phagocytes per c.c. in a serum diluted twenty or thirty times. Can any conditions less like these obtaining in the human organism easily be conceived ? At the same time it must, however, be admitted that under certain conditions this ' dilution

method ' gives a more accurate idea of the total amount of protective substances present in the blood-serum.

The method suggested by Stewart,[1] Dodds,[2] and Veitch[3] has, on the other hand, much to recommend it, and most nearly of all approaches the natural conditions. It is conducted as follows : One volume of blood is withdrawn from the patient in a sterilized capillary pipette, and at once thoroughly mixed with an equal volume of 2·0 per cent. solution of sodium citrate in 0·8 per cent. salt solution, and the ends of the pipette sealed. The same is done with the control normal blood. These mixtures are preserved till required, and, according to Stewart, keep unaltered for twelve hours, or, if kept in a refrigerator, for three days.

The phagocytic mixture is made by taking two volumes of the blood citrate mixture and one volume of the bacillary emulsion, and proceeding in the usual way. The hæmophagocytic index thus determined agrees well, as a rule, with the opsonic index, while the method affords a certain saving of time, especially when only two or three bloods have to be examined, and is more accurate, inasmuch as clumps of leucocytes very rarely occur. The employment of the blood-plasma instead of serum, and of the patient's own leucocytes, are two important modifications; for, as Dreyer and Ainley Walker[4] have shown, the plasma is usually more highly agglutinative than the serum and less rich in complement ; inequality of opsonic content in the two is therefore also likely. As regards the leucocytes, Rosenow,[5]

[1] *Journal of Bacteriology*, 1908.
[2] *British Medical Journal*, October 12, 1907, p. 948.
[3] *Journal of Pathology and Bacteriology*, January, 1908, p. 353.
[4] *British Medical Journal*, January, 1909, p. 151.
[5] *Journal of Infectious Diseases*, 1906, vol. iii., p. 683.

Glynn and Yorke,[1] Bushnell, and others, have shown that these are by no means an indifferent factor, the phagocytic power of some being above normal, and of others below. This is especially true in the case of pneumonia, where Rosenow found that the leucocytes were, as a rule, hyperactively phagocytic. Ledingham and Bulloch observed, on the other hand, a marked diminution of phagocytic power in the leucocytes during the period of leucocytosis produced by injection of sodium cinnamate.

[1] *Lancet*, September 19, 1908.

CHAPTER III

PREPARATION OF THE VACCINE

THE general consensus of opinion is that the best possible results are, as a rule, only to be looked for when organisms isolated from the patient's own lesion are employed for the manufacture of the vaccine. Several considerations may, however, militate against the advisability of this procedure. The chief of these are as follows :

1. Where the isolation of the organism is so difficult and tedious that the resultant loss of time would fail to compensate for the advantages obtained. An excellent example of this is afforded in tuberculous affections. Here we are compelled to resort to inoculation experiments, the animals usually selected for the purpose being the rabbit or guinea-pig, and the site of inoculation either the subcutaneous tissue of the groin of the latter or the anterior chamber of the eye of the former. Of these two animals, the guinea-pig is generally held to be the more susceptible to the tubercle bacillus, dying of general tuberculosis from six to ten weeks after inoculation, according to the virulence of the organism and the number introduced. On the other hand, if tuberculous material be introduced into the anterior chamber of the eye of the rabbit, an iritis which is almost pathognomonic results in from two to four weeks. In either case the loss of time is very considerable. Nor is this all. The growth of the tubercle bacillus is again so slow, and the preparation of

tuberculin so difficult an operation, that another two or three months would be consumed in the preparation of the vaccine. This is very greatly to be regretted, for many of the only partial successes or even failures in cases treated by tuberculin are possibly very largely due to the employment of stock tuberculin. This question will be again referred to later.

A second example of this class of case is afforded in some chronic gleets. The gonococcus may be visible in smears of the urethral secretion, yet, despite the utmost care in taking the cultures, it may prove impossible to free the gonococcus from the contaminating organisms.

2. The infection, although localized, may be of so acute and destructive a type that the loss of even one or two days may be of vital importance. An excellent example of this is seen in gonorrhœal conjunctivitis in the adult. Here prompt injection of a stock vaccine is obligatory immediately the patient is diagnosed. I have seen cases, so severe that total destruction of the sight was inevitable in two or three days, thereby completely held in check, and, save for the destruction which had already occurred, cured within a week (*vide* chapter on The Eye).

3. Where the infection is so very chronic that it is reasonable to suppose that the virulence of the infection has been greatly reduced, though even here it is better, wherever possible, to test the virulence by an inoculation experiment upon animals. Good examples of this class of case are afforded by—(*a*) very chronic cases of osteomyelitis which have been subjected to considerable surgical treatment; (*b*) chronic gonorrhœal infections, especially old gleets in the male, and tubal cases in the female.

4. A final exception may be made in the instance of such organisms as seem to be definite entities, and not to