IMPROVED METHODS OF STANDARDISING BACTERIAL VACCINES IN HÆMOCYTOMETER CHAMBERS.¹

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THE simplest scientific method of standardising bacterial vaccines is the direct enumeration of the bacteria in some form of hæmacytometer chamber. It was first suggested in 1908 by Mallory and Homer Wright,² who employed a well 0·02 mm. deep, an optically plane coverslip, a 1/6-inch dry lens, and no staining fluid; also by Leith Murray³ (1908), who used a common Thoma-Zeiss hæmacytometer with a well 0·1 mm. deep, an ordinary coverslip for microscopical work which, though specially chosen, is not optically plane, a 1·12th oil-immersion lens, and a staining solution of weak Giemsa. Owing to certain defects in these methods Glynn and Cox⁴ in January, 1911, advised using the Helber chamber 0·02 mm. deep with a special optically plane rigid coverslip 0·18 mm. thick, and a diluting fluid of weak carbol thionin. The Helber chamber was originally designed for the enumeration of blood plates; it and the cover-slips are made by Zeiss. Obviously as every laboratory worker possesses a Thoma-Zeiss hæmacytometer it is more economical to use this same apparatus for standardising vaccines. But for reasons to be subsequently stated the Thoma-Zeiss chamber, which is 0·1 mm. deep, is less suitable than the Helber chamber, which is only 0·02 mm. deep. Both these pieces of apparatus, however, give more accurate results than Wright's method of mixing measured volumes of bacterial emulsion and normal blood. When standardising bacteria with a hæmacytometer chamber it is essential in order to diminish eye strain and increase accuracy first to stain the bacteria, and second to count them with an oil-immersion lens. The ordinary coverslips supplied with a Thoma-Zeiss chamber are too thick to permit the use of an oil-immersion lens, and it has been necessary to devise a new coverslip suitable for use with most oil-immersion lenses.

Three points, therefore, have been considered: (1) The most suitable staining fluid for diluting the bacterial emulsion in a hæmacytometer pipette; (2) the most suitable optically plane rigid coverslip for use with the Thoma-Zeiss hæmacytometer chamber 0·1 mm. deep and an oil-immersion lens; and (3) the most suitable form of Helber chamber 0·02 mm. deep and optically plane rigid coverslip for use with an oil-immersion lens.

Staining of the bacterial emulsion.—After trying various methods we recommend a diluting fluid for the hæmacytometer pipettes of weak carbol thionin prepared as follows:—Stock Solution A: saturated thionin blue (Grubler) in absolute alcohol. Stock Solution B: 1 per cent. of pure carbolic acid in clear tap water. Before use take 1 part of Solution A to about 40 of B for most organisms, or 1 part of A to about 20 of B for streptococci. The mixture will keep a considerable time, but should always be filtered before use; frequent filtration, however, may weaken the stain unless the filter paper is saturated with thionin. It is important to keep the bacterial emulsions, stock solutions, counting chamber, and pipettes absolutely free from dust, particles of cotton-wool, &c. The most satisfactory method of preparing an absolutely dust-free carbolic acid solution is to allow a large quantity to stand for a few days in a glass measuring cylinder, when the dust sinks to the bottom or rises to the top. The capacity with which bacteria take up the carbol-thionin stain varies somewhat with the type and sometimes the strain of the organism, or with the age of the culture. Streptococci require stronger stain than most bacteria. If the proportion of thionin is too high the bacteria are stained almost instantaneously, and an abnormal number adhere to the under surface of the coverslip; agglutination also occurs.

Agglutination is always present if too much thionin is employed. We have found by actual experiment that it does not depend upon the small quantity of alcohol or carbolic acid present in the solution, but upon the intensity of the staining. Agglutination will also occur if the diluting pipette has been washed out with absolute alcohol and is not properly dried before use. We are convinced that carbol thionin is superior to any other stain as methylene blue, fuchsin, or even the fashionable Giemsa. It stains more intensely and is quite free from precipitate; further, the proportion of thionin can easily be adjusted to the staining capacity of the organism. The great defect of the Giemsa solution is the tendency of the stain to precipitate even after filtration if the amount of Giemsa is strong enough to more than faintly colour the bacteria. We have been unable to remove the precipitate by substituting prolonged centrifugalisation for filtration.

Brownian movement may be troublesome. This, as can easily be demonstrated, is always more marked with *strong* emulsions, or when the bacteria are faintly stained; it ceases completely when the stain is strong enough to cause some agglutination, but agglutination is undesirable. Brownian movement can be avoided in properly stained emulsions by not having more than an average of about half a dozen bacteria per square; a much larger number, even if motionless, is difficult to count. The movement is more troublesome in counting chambers 0·1 mm. than 0·02 mm. deep.

Bacteria adhering to the under surface of the coverslip.—A certain proportion of the bacteria always adhere to the under surface of the coverslip and the number is increased by overstaining. In a series of over 100 observations made by the different observers it was found that the average number of staphylococci and colon bacilli adhering to the under surface of the coverslip was about 15 per cent. One point must be alluded to in the enumerating of bacilli like typhoid or colon—viz., they not infrequently stand at right angles to the coverslip or floor of the chamber, and consequently appear as round blue dots.

¹ Abstract of portion of a paper entitled "Observations upon the Standardisation of Bacterial Vaccines by the Wright, the Hæmacytometer, and the Plate Culture Methods," Journal of Pathology and Bacteriology, 1914, vol. xviii., pp. 379-400.

² Mallory and Homer Wright (1908): Pathological Technique, Saunders, fourth edition, p. 457.

³ Murray, Leith: THE LANCET, March 14th, 1908, p. 790.

⁴ Glynn, E. E., and Cox, G. L.: Journal of Pathology and Bacteriology, 1911, vol. xv., p. 360.

Standardisation in the ordinary hæmocytometer chamber 0.1 mm. deep.—The vaccines were standardised before sterilisation in case the heating for half an hour at 60° C. produced agglutination. The optically plane polished coverslip supplied with the apparatus is too thick for use with an oil-immersion, or even with most types of 1/6th inch dry lenses. Leith Murray in 1908 to remedy this defect suggested using a thin coverslip chosen from those supplied for ordinary microscopical work in the following manner. "A series of slips is cleaned and applied successively to the cleaned and dried plate-glass slip provided with the apparatus after breathing on the opposing face of each. Pressure is applied to make them adhere, and the slips which show Newton's rings well distributed over the surface are selected for use." This ingenious method, however, is not satisfactory if scientific accuracy is demanded. Many oil-immersion lenses depress such a coverslip and consequently diminish the depth of the counting chamber; thus a No. 2 coverslip of about 0.17 mm. is too thick for an ordinary 1/12th achromatic oil-immersion lens supplied by Leitz, Swift, or Beck with a free working distance of 0.17 mm., while even a No. 1 coverslip of about 0.12 mm. is too thick for an

enumerating red blood corpuscles or leucocytes; why, then, should it be employed for the standardisation of bacterial vaccines when accuracy is of even more importance? Zeiss manufacture an optically plane cover-glass for counting chambers of 0.18 mm. thick, strengthened with a "cemented cylindrical glass cell." "The lower side of this cover-glass is polished accurately plane." This slip gives Newton's rings without difficulty. We have found, however, by actual experiment that it is too thick for all ordinary oil-immersion lenses when used with a hæmocytometer chamber 0.1 mm. deep. In order to understand the reason of this it is necessary to discuss the free working distance of an oil immersion lens.

Free working distance.—Zeiss⁵ has defined the free working distance "as the distance between upper surface of a cover-glass 0.17 mm. thick and the lower surface of the lens mount, the lens being sharply focussed for an object situated immediately below the cover-glass." The free working distance of a Zeiss achromatic 1/12th oil immersion lens when oil is employed is stated to be 0.15 mm., thus the "actual distance" between the metal mount of the front lens and the object is 0.32 mm. Fig. 1 shows that a lens with a free working

FIG. 1.

FIG. 2.

FIG. 3.

Diagrams illustrating free working distance of an oil-immersion lens and two types of hæmocytometer. Fig. 1. Illustrating free working distance. Fig. 2. Showing ordinary 1 mm. hæmocytometer chamber with new 0.13 mm. coverslip. Fig. 3. Showing new 0.02 mm. chamber with wide safety trench and 0.18 mm. coverslip, recommended by authors.

achromatic 1/12th supplied by Zeiss or Bausch and Lomb with a free working distance of 0.15 mm.

The pressure of the lens upon the coverslip can easily be demonstrated by the to-and-fro movement of the bacteria in the counting chamber which occurs with slight alteration in the fine adjustment. Even if the lens does not actually touch the coverslip, alterations in the fine adjustment may cause movement of the bacteria in the counting chamber from suction or adhesion between the two surfaces owing to the immersion oil. This suction effect may be very marked with thick oil and certain types of lens mount. It can easily be demonstrated in another way by taking an ordinary microscope slide, placing a drop of oil in the middle, and pressing the lens down gently till it almost touches the glass. If the lens is quickly raised the slide will be lifted with it. This suction action can be diminished if very thin immersion oil is employed.

Another point may be alluded to. Even if a thin coverslip of 0.15 mm. or less does give Newton's rings in some part when pressed down upon a moistened glass plate, it by no means follows that the surface is mathematically plane when a large portion forms the roof of a counting chamber. The elasticity or want of rigidity of such a coverslip, when in position over the filled chamber, may be easily appreciated by making a very gentle pressure upon its centre with the point of a pencil. This will cause the fluid to bulge over the edge of the chamber. No one would think of using an ordinary microscopical coverslip which is neither rigid nor optically plane for

distance of 0.1 has an actual distance of 0.27. It is important to note, however, that these figures are approximate because the "metal mount of a front lens is necessarily subject to unavoidable slight variations" (Zeiss⁶). The free working distances of a 1/12th oil-immersion achromatic objective supplied by other makers are as follows: Ross, 0.15 mm.; Swift, 0.17 mm.; Watson, 0.17 mm.; Baker, 0.17 mm.; Leitz, 0.17 mm.; Reichert, 0.15 mm.; Bausch and Lomb, 0.15 mm.; Zeiss, 0.15 mm. The free working distance of many apochromatics is frequently much less than 0.15, but apart from their expensiveness they are hardly suitable for such work as the standardisation of vaccines owing to the greater care required in using them. Leitz manufacture a 1/12th oil-immersion fluorite objective 1.32 N.A., and Watson a 1/12th with a free distance of 0.12 and 0.09 mm. respectively.

These figures indicate at first sight that the free working distance of the ordinary 1/12th immersion lens likely to be used in the laboratories with the exception of the special lenses made by Leitz and Watson is theoretically never less than 0.15 mm. Unfortunately, we found by actual experience when working with coverslips of known thickness that the free working distance of many lenses is in reality very much less than stated in the catalogues. Thus a well-known firm tested one of their own new lenses with a reputed free working distance of 0.17 and found it only 0.10, while a lens by another maker stated to be 0.15 was only 0.08. The free

⁵ Zeiss, 1906, Catalogue, thirty-third edition, p. 16.

⁶ Ibid.

working distance of a lens partly depends upon the refractive index of the medium—air, water, or oil—between its under surface and the object examined. The greater the refractive index, the greater its free working distance. Thus, the free working distance of a 1/12th Zeiss oil-immersion lens is much less than 0.15 mm. if no oil is used, as the refractive index of oil is 1.515 but that of air only 1.

Professor Wilberforce kindly ascertained that the refractive index of the watery carbol-thionin solution we employ was exactly the same as that of water, and may be taken as 1.333; the refractive index of the immersion oil supplied by Zeiss is 1.515. The substitution of 0.1 mm. of water for 0.1 mm. of oil will diminish the free working distance of the oil-immersion lens according to the fraction

$$\frac{0.1 \times 1.515}{1.333} = 0.1136 \text{—i.e., } 0.1 \text{ mm. of carbol thionin}$$

is equivalent to 0.114 mm. of immersion oil—and the lens must be brought 0.014 mm. nearer the object than it otherwise would have been if the chamber contained oil instead of water (Fig. 2). After a large number of experiments we have found that cemented coverslips of 0.18 mm. and 0.15 mm. are too thick for a 0.1 mm. well, while those of 0.10 mm. are liable to warp after much use. Warping of the coverslips can easily be demonstrated by observing the images of the window frame reflected from its surface, when it will be noted that the lines of the frame are no longer straight.

We have found, however, that a coverslip 0.13 mm. thick is not only durable but also suitable for all achromatic oil-immersion lenses stated to have a free working distance of 0.17 mm. and for those by most makers stated to have a free working distance of 0.15 mm. The lens already alluded to with a reputed free working distance of 0.15 mm., but when actually tested of only 0.08 mm., could not be used with a 0.1 mm. chamber without causing movement of the bacteria floating in the fluid. As the lens mount could not touch the coverslip, being 0.006 mm. distant, the movement of the coverslip is explained by the immersion oil causing adhesion between it and the lens mount. In order to make full allowance for the over-estimation of the free working distance by some makers we have illustrated diagrammatically in Fig. 2 a lens with a free working distance of 0.1 mm. used with a hæmocyto-meter chamber 0.1 mm. deep. This lens has a corrected free working distance of 0.026, which is ample for ordinary purposes.

Messrs. Zeiss make such a coverslip, price 3s., which can be sterilised in formol or corrosive sublimate solution; but not with alcohol or xylol, for these substances dissolve the cement and cause warping. We have found these coverslips still optically plane after several months' use; they are easily repaired if the cement is damaged.⁷

Standardisation with a hæmocyto-meter chamber 0.02 mm. deep.—The easiest and best method of eliminating the defect of the small free working distance of an oil-immersion lens is to diminish the depth of the counting chamber instead of diminishing the thickness of the coverslip. After experimenting with specially made counting chambers of various depths we have come to the conclusion that a 0.02 mm. chamber with an optically plane cemented coverslip 0.18 mm. thick as catalogued by Zeiss is the best, and suitable for all practical purposes. This apparatus can be used

with all achromatic oil-immersion lenses, even with one by Watson N.A. 1.30, having a free working distance of 0.09 mm.

Fig. 3 shows that a lens with a free working distance of 0.1 mm. used with such a counting chamber and coverslip has the corrected free working distance of 0.068 mm., which is ample. The only disadvantage of the 0.02 counting chamber was the difficulty of spreading the drop, especially if the glass is slightly greasy. We have completely remedied this defect by increasing the width of the trench from the usual 0.15 mm. to 2.5 mm., for this provided a larger cistern for the overflow of surplus when the drop is a little too big. (See Fig. 4.) If,

FIG. 4.

1 and 1A.—Ordinary 1 mm. hæmocyto-meter chamber with new 0.13 coverslip suitable for all immersion lenses with a F.W.D. of 0.17 mm. and some of 0.15 mm. 2 and 2A.—New 0.02 mm. chamber with wide safety trench and coverslip 0.18 mm. thick, suitable for all immersion lenses with a F.W.D. from 0.17 mm. to 0.09 mm. (Note.—Too large a drop of methylene blue has been placed in 0.02 chamber and the excess is bulging over into the trench, but owing to its extra width the fluid has not touched the opposite wall, and so run under the coverslip by capillary attraction, and increase the depth of chamber.)

however, the surplus of fluid is so great that it comes in contact with the opposite wall of the trench, then, as is well known, it spreads by capillary attraction everywhere under the coverslip, Newton's rings disappear, and the depth of the chamber is increased.⁸

Calculation with a 0.02 mm. chamber, the number of bacteria per square is multiplied by 20,000 instead of 4000. Bodley Scott⁹ has recently recommended a counting chamber 0.01 mm. deep and an optically plane coverslip, though he does not state how such a coverslip is obtained. The disadvantage of such a counting chamber ten times shallower than the ordinary Thoma-Zeiss and twice as shallow as the Helber are two: (1) The greater the difficulty in spreading the drop accurately; and (2) any error in the depth of the

⁷ Zeiss now make a 3.5 mm. achromatic oil-immersion lens with a free working distance large enough to take a 0.1 mm. chamber with a coverslip 0.18 mm. thick.

⁸ This apparatus will be catalogued by Zeiss under the name of the Helber-Glynn chamber, price 15s.

⁹ Scott, T. Bodley, and Scott, G. Bodley: THE LANCET, Sept. 23th, 1912, p. 879.

chamber or in the correct adjustment of the coverslip is more serious.

Settling of the bacteria in the counting chamber.—One slight disadvantage of the chamber method is the difficulty of counting the bacteria when first introduced owing to their movement. After a few minutes, however, the great majority settle at the bottom of the chamber and remain motionless, though some may continue in Brownian movement indefinitely. Obviously the settling will take longer in a 0.1 mm. than in a 0.02 mm. chamber. A large number of observations made upon the different strains of colon bacillus and staphylococcus indicated that whereas in a 0.02 mm. chamber the great majority of the bacteria had settled in 15 minutes, in a 0.1 mm. chamber a considerable number were still moving after half an hour. A further series of observations showed that quite accurate accounts could be made with a 0.1 mm. chamber after the contents had settled for 15 minutes, provided that great care is taken to hunt for floating organisms. Hunting for the floating bacteria takes a considerable time if many are present, far longer than in a 0.02 mm. chamber. Another advantage of the 0.02 mm. chamber is the greater facility with which the bacteria adhering to the under surface of the coverslip may be counted. First, because only a slight turn of the fine adjustment is necessary to focus them; and second, because the outlines of the squares are still seen when the bacteria are focussed, and consequently there is no difficulty in ascertaining to which squares they belong.

Summary of the advantages of the 0.02 mm. chamber.—Obviously it is more economical to standardise vaccines in the ordinary hæmocytometer counting chamber 0.1 mm. deep, but the 0.02 mm. chamber is preferable for the following reasons: (1) The apparatus is suitable for all achromatic oil-immersion lenses, even if the free working distance is as low as 0.09; (2) the coverslip being 0.18 mm. thick is more durable; (3) almost all the bacteria settle at the bottom of the chamber in 15 minutes when accurate counts can be made, whereas in the 0.1 chamber a considerable number are still moving after half an hour; (4) bacteria adhering to the under surface of the coverslip and any still floating in the chamber are much more easily enumerated; (5) the optical definition of the bacteria is better owing to the smaller quantity of fluid; and (6) the difficulty of accurately adjusting the drop of emulsion in such a shallow chamber has been entirely removed by widening the safety trench for holding surplus fluid to 2.5 mm.

The relative accuracy of the Wright and counting chamber methods for standardising vaccines.—It is now 12 years since Wright¹⁰ introduced his simple and ingenious method of standardising vaccines. Though the method has been of great service to vaccine therapy we are convinced it suffers from two serious defects, first, it is usually very inaccurate, and second, it usually seriously underestimates the strength of the bacterial emulsion. In order to ascertain whether the Wright or the chamber method gives the more accurate results, two observers independently standardised bacterial emulsions by each method, and the *percentage deviation from the arithmetic mean* of their counts was taken as a measure of the error in the method. This is a more practical test of the accuracy than that obtained by two observers

enumerating the bacteria in the same series of squares or microscopical fields. Forty-four bacterial emulsions made from different strains of colon bacillus, staphylococcus and streptococcus were tested in duplicate in this manner. We found that the average error was less than 5 per cent., with a minimum of 0.5 per cent. and a maximum of 21 per cent. for the chamber method, and more than 15 per cent., with a minimum of 1.1 per cent. and a maximum of 54 per cent. for Wright's.

The error with a 0.1 mm. chamber, though a little more than with a 0.02 mm., is also much less than in Wright's method. In six bacterial emulsions of colon bacillus and staphylococcus standardised in a 0.02 mm. and 0.1 mm. chambers and by Wright's method the average error was 3.1 per cent., 5.4 per cent., and 34.1 per cent. respectively. The average time occupied in counting these emulsions in the 0.02 mm. chamber (64 squares) was 8 minutes, in the 0.1 mm. chamber (64 squares) 10 minutes, with Wright's method (50 fields) 12½ minutes. This further demonstrates the superiority of the hæmocytometer method, especially the 0.02 mm. chamber.

Wright's method usually underestimates the strength of the bacterial emulsions.—This serious defect has been attributed to various causes. In our opinion the chief are, first, a large number of isolated bacteria are usually left behind at the beginning of the smear, as we have shown elsewhere; second, small groups of bacteria mixed with blood platelets and leucocytes are drawn to the end of the film, where they are usually unnoticed unless specially looked for with a low power. In both cases these bacteria almost certainly escape enumeration. It is no use trying to remedy this defect of unequal distribution of the bacteria and red cells by counting indiscriminately fields selected from all parts of the smear.

A series of emulsions were standardised by the 0.02 mm. chamber and Wright's method. We found that the average percentage increase in 15 staphylococcus emulsions standardised by the 0.02 mm. chamber over Wright's method was 76 per cent., with a maximum of 192 per cent.; and in 12 emulsions of colon bacillus the average increase was 72 per cent., with a maximum of 228 per cent. Additional figures are given elsewhere. Rarely the chamber method gave a lower count than Wright's. This is only to be expected on account of the unequal distribution of bacteria and red cells.

Summary and Conclusions.

1. The most scientific method of enumerating the bacteria for vaccines is in some form of hæmocytometer chamber.

2. An optically plane and rigid coverslip must be used with such apparatus, for it is recognised as essential when counting blood and is still more essential when counting bacteria, where accuracy is of greater importance. Owing to the small free working distance of oil-immersion lenses most optically plane coverslips are too thick.

3. Two types of hæmocytometer chamber may be employed: (a) The ordinary chamber 0.1 mm. deep, with an optically plane coverslip 0.13 mm. thick. This is suitable for all oil-immersion lenses with a free working distance of 0.17 mm. and some of 0.15 mm. (b) A chamber 0.02 mm. deep, with a special safety trench 2.5 mm. wide and optically plane coverslip 0.18 mm. thick. This is suitable for all achromatic oil-immersion lenses, even if the free working distance is as low as 0.09.

4. The 0.02 mm. chamber is much superior for the following reasons: (a) Almost all the bacteria

¹⁰ Wright: THE LANCET, July 5th, 1902, p. 11.

settle at the bottom in 15 minutes, when accurate counts can be made, whereas in the 0.1 mm. chamber a considerable number are still moving after half an hour. (b) Bacteria adhering to the under surface of the coverslip or still floating in the chamber are more easily enumerated. (c) The optical definition of the bacteria is better owing to the smaller quantity of fluid. (d) The free working distance is greater, and the coverslip being 0.18 mm. thick is more durable. (e) Counting is quicker owing to the much smaller number of moving bacteria and the greater ease in focussing the top and bottom of the chamber.

5. A weak solution of carbol thionin is the best diluting and staining fluid; unlike a weak solution of Giemsa it is quite free from precipitate and stains more intensely.

6. Enumeration of stained bacteria in a hæmocytometer chamber, particularly in one of 0.02 mm. deep, is more rapid and much more accurate than by Wright's method.

7. The hæmocytometer method does not underestimate the strength of the bacterial emulsion as usually happens with Wright's method, sometimes to a very serious extent.

8. This research has been undertaken because we believe it is the duty of the bacteriologist to standardise and dispense his vaccines with the same accuracy and care as that exercised by the pharmacist in standardising and dispensing his drugs. Scientific vaccine therapy will be advanced by scientific exactness.

A METHOD OF INCREASING THE ACCURACY AND DELICACY OF THE WASSERMANN REACTION.

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WE bring forward in the following article our system for performing the Wassermann reaction in detail and discuss the various objections that might be raised to our methods of increasing its accuracy and delicacy. We strongly urge that in performing the Wassermann reaction the substance used as "antigen" should be purely "antigenic" and freed from complicating anticomplementary and hæmolytic factors.

The Antigen.

An ox heart is trimmed free from fat, &c., finely minced, allowed to stand at room temperature for 24-36 hours, dried in a current of warm air in about four hours, and finally completely dried in an evacuated desiccator over sulphuric acid. It is then finely powdered and extracted with 3-4 volumes of absolute alcohol at 37° C. for three days, then the extract is filtered off and fresh alcohol added, and the extraction continued for another three to four days. The united extracts are evaporated *in vacuo* at 56° C. in a distilling apparatus. (The evaporation *in vacuo* is very rapid, so that a Winchester quart is evaporated in about two hours.) The residue is then extracted with 0.730 ether. The ethereal extract is next precipitated with acetone, the precipitate collected, washed with acetone, redissolved in ether and

reprecipitated with acetone. This precipitation and re-solution is continued till the precipitate and solution are quite white. The precipitate is collected, washed with acetone, pressed on filter paper, and is ready for use.

It will be seen that the precipitate contains two phosphatid fractions—viz., the free and bound N_1P_1 fractions. The N_1P_2 fraction is not extracted to any appreciable degree; the N_2P_1 fraction is not taken up in the ethereal extract of the alcohol residue. There may be a little jecorin of Drechsel, but very little. After saponification of a large quantity of this "purified" antigen we have been unable to demonstrate even traces of cholesterol. By the "ninhydrin test" our antigen always gives a marked α -amino-acid reaction. It appears probable to us, from work that is in progress, that the antigenic property of a phosphatid may depend upon the presence of protein degradation bodies in combination with the phosphatid. For example, the Cuorin fraction N_1P_2 of a perfectly fresh tissue does not give the ninhydrin reaction and does not act as an antigen, whereas the same fraction extracted from a suitably autolysed tissue gives both the ninhydrin reaction and acts as a good antigen.

Three grammes of the purified phosphatid are dissolved in the least possible quantity of ether and then made up to 100 c.c. with absolute methyl alcohol (Merk) and stored in a well-stopped, brown-tinted glass bottle at laboratory temperature. This stock solution, if kept free from moisture, will keep its titre absolutely constant for over one year. The slight deposit that may occur after it is first made up does not appreciably alter the titre, and need not be removed. For the purpose of the test 1 c.c. of this solution is poured on to 9 c.c. of normal saline and the whole rapidly shaken to make the emulsion.

The emulsion is then standardised with regard to its hæmolytic, anticomplementary, and antigenic properties as described subsequently. If the phosphatid shows *any* hæmolytic or anticomplementary properties in 500 c.mm. of the saline emulsion we consider it to be unsuitable for the Wassermann reaction, and continue the process of purification until these properties are removed. The antigenic value should be in 2 or 1 c.mm. of the saline emulsion (= 3 millionths of a gramme of the original phosphatid), which strength can be readily obtained. The stock solution should be standardised once a month.

Standardisation of an "Antigen."

For this, three factors have to be taken into consideration as was first pointed out by Noguchi: (1) hæmolytic; (2) anticomplementary; and (3) antigenic.

1. *Hæmolytic factor.*—To each of a series of five tubes containing 1 c.c. of normal saline 100 c.mm. of 10 per cent. red cell suspension are added and then 500, 400, 300, 200, 100 c.mm. of the antigenic emulsion respectively. The tubes are shaken and placed in the water-bath¹ at 38° C. for three hours, at the end of which time the presence of hæmolysis is noted.

2. *Anticomplementary factor.*—To each of a series of 9 tubes containing 1 c.c. of normal saline $1\frac{1}{2}$ units of complement are added, and then 500, 400, 300, 200, 100, 80, 60, 40, 20 c.mm. of the antigenic emulsion respectively. The contents of the tubes are mixed and incubated for one hour in the water bath at 38° C. At the end of this time $1\frac{1}{2}$ units of amboceptor and 100 c.mm. of 10 per cent. red cells are added. The contents are remixed and the tubes returned to the water bath for half an

¹ A convenient bath (thermostat) with racks can be obtained from Messrs. Baird and Tatlock.