REVIEW ARTICLE

MATERIALS USED IN GREAT BRITAIN FOR THE ACTIVE IMMUNISATION OF MAN AGAINST DIPHTHERIA

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Following the introduction of antitoxin for the treatment of diphtheria in 1895 there was a fall in the case-fatality in these islands which, some ten years later, had reached about one-third of that of pre-antitoxin days. Although the rate declined a little further, and fluctuated from year to year, it persisted at such a level that diphtheria remained as a major concern for public health officers and the authorities of infectious diseases hospitals, calling for a sustained and intensive effort in the field by the former and the maintenance of an expensive hospital service to provide for the latter. In spite of all the measures taken, however, in the ten years prior to the outbreak of the second world war, there were from 2,500 to 3,000 deaths from diphtheria every year in this country, and this rate continued until 1941. In 1947, however, the deaths had reached the low record—up to then—of 244 for that year, and the (uncorrected) figure for 1948 is even lower at 156. Since a decline in case-fatality rate may be independent of measures taken for prevention or treatment, it may be prudent to reserve a final judgment to a more detailed examination of all the factors concerned; but the evidence at present available is that active immunisation has contributed very materially to the extremely low incidence and mortality from diphtheria in recent years, and many take the view that this has been the dominating factor.

The materials which have been used, and have thus made their contribution to this outstanding achievement of public health endeavour, have attracted the continued interest of research workers in English laboratories from the early 1920's and the preparations used, in this country exclusively and in other countries to a considerable extent, are largely the fruits of their labours. Accordingly, it may be of interest to those whose duty it is to supply these materials, to keep them before the notice of our people and advocate their continued employment in the future, to know something of their historical development, their nature, preparation, composition, properties and use.

Although the value of antitoxin for prophylaxis and therapy was recognised, it was obvious that protection from attack was of greater importance than the treatment of individual cases and the passive immunisation of contacts: so long as susceptible populations remained

unprotected, the occurrence of cases and epidemics was a constant threat to life, especially of young children, and to the public health and economy.

Since it was known that susceptible animals could be successfully immunised against diphtheria there did not seem to be any reason why a similar result should not be achieved with man. The pathology of the disease indicated that the aim should be to provide protection against the characteristic toxæmia, and it is not surprising that the first attempts were on similar lines to those which had succeeded with animals, i.e., by the injection of toxin. Accordingly in the early years of this century there are a few records of the immunisation of man with small but increasing doses of toxin: the local reactions and the pain at the site of injection were reported. Attempts were also made to immunise man by the injection of bacterial vaccines. None of these measures, however, appeared very promising, and the success which has been achieved is a result of investigations on different lines.

In the United States, Theobald Smith[1,2], who then held important appointments at Harvard and the Antitoxin Laboratories of the Massachusetts State Board of Health, observed that the guinea-pigs supplied for his use were immune to diphtheria: on investigation he found that they, or their progenitors, had been used for testing diphtheria antitoxin, the injection of mixtures of toxin and antitoxin having served to immunise them. This observation was the starting point of a series of important researches concerning the properties and immunological behaviour of neutral, over-neutralised and under-neutralised mixtures in guinea-pigs and of the transmission of antitoxin, and in 1907 Theobald Smith advocated the use of certain of these mixtures for the active immunisation of man. It seems strange that it was not until 1913 that this suggestion, coming from so eminent and recognised an authority, was given practical application. The facts were not in question because many workers had shown that antitoxin was produced in animals which had survived the injection of mixtures of toxin and antitoxin. There appear to have been two main reasons for this delay; Park[3], who was then and for many years afterwards the acknowledged leader in this field and was always ready to exploit any discovery or advance in the laboratory which promised to help in his fight against diphtheria in New York, described his own investigation of these mixtures in guinea-pigs. He observed that the mixtures which would have to be used for the effective immunisation of man were toxic for guinea-pigs and, quite clearly, Park was hesitant about injecting material into children which caused paralysis in guinea-pigs. Another deterrent was that, at that time, there was no simple means of finding out, first, which members of a community needed protection and, second, whether the measures taken had succeeded or failed.

In 1913, however, two papers appeared in Germany which removed these doubts. Schick[4], basing his observations on the earlier work of Römer[5], described his test by the application of which, it was claimed,

a population could be divided into susceptible and immune persons: and Behring[6,7] reported that mixtures of toxin and antitoxin could safely be injected into children. A certain mystery surrounded the manner of preparation and the composition of these early mixtures of T.A., as they came to be known, but it appears that the German workers were feeling their way and testing the antigenic efficiency, and the toxicity, of mixtures of different composition. Large numbers of children were inoculated in the Magdeburg district of Germany; the results were such that any remaining doubts in Park's mind regarding their safety were dispelled and, armed with this knowledge and with the Schick Test as a helpful guide, the way was clear for the application of these important advances in the United States. It is interesting to note that, while Park deprecated Behring's claim that these T.A. preparations were a new discovery, as they had been used extensively for the immunisation of animals, he emphasises the importance, to the campaign for active immunisation of man, of his demonstration of their safety.

Since its introduction the Schick Test has been the subject of much controversy, and the manner of its application, its interpretation, its value as an aid to diagnosis and as a guide for administrative action have all been challenged. These controversies cannot be discussed here, but the practical importance of the test at the time it was first applied can hardly be exaggerated. It gave confidence to field workers who were dealing with potentially dangerous materials of which they had little knowledge or experience; and, as these increased, the Schick Test became an almost essential part of the mechanism by means of which active immunisation of human populations has been achieved. One ground for criticism, however, has deserved more attention and consideration, in countries other than this, than it has actually received. Schick considered that the quantity of toxin contained in the Schick Test Dose was one-fiftieth of the minimum lethal dose of a matured toxin, as determined by injection into guinea-pigs. This definition is unacceptable on two grounds. Trevan[8] pointed out that the term " minimum lethal dose " has no clear or proper meaning and indicates a vague quantity the actual determination of which is of doubtful accomplishment; and, even if it were permissible to define the Schick Test dose in terms of toxicity alone, the amount should be expressed in terms of the quantity found to be lethal for a stated percentage of animals as, for example, the LD50 dose; this quantity has a meaning and can be determined with an accuracy dependent on the number of animals used. An even more serious objection to Schick's original definition was pointed out by Glenny[9] and independently by Kellog[10]: since the test determines the approximate concentration of antitoxin its definition must be expressed in terms of combining power for antitoxin and not only in terms of toxicity—the latter merely ensuring the presence of an adequate, but not too large, quantity of toxin for injection into human beings. The absurdity to which the test can be reduced by limiting its definition to toxicity was shown by Glenny and Waddington[11], who prepared a series

of Test Toxins, each conforming to Schick's original definition in that the dose, in each case, contained the amount of toxin postulated by Schick as the all important factor, but their combining powers varied to such an extent that they indicated antitoxin concentrations varying from one half of that usually detected by Schick Test Toxin to 200 times that level. It is difficult to understand why Schick Test Toxins should not be standardised on sound principles, based on a stable standard and thus reproducible and comparable the world over, especially as this could be done most economically, partly by means of the flocculation reaction and, in any case, by the use of a very few animals. This is not merely an academic question raising issues of no practical importance: how much of the controversies which have raged over the Schick Test in past years may have been due to faulty standardisation based on improper principles cannot be assessed; but the question is not without importance to problems which may arise in the future. It is quite likely that it may be necessary to estimate higher antitoxin concentrations in human populations than that hitherto indicated by the Schick Test Toxin as at present prepared for issue: this could be done without difficulty by selecting, or preparing, a Test Toxin having the desired combining power for antitoxin: its suitability for use, as indicated by this property and its content of free toxin, is easily checked by the intracutaneous injection of mixtures of the sample with antitoxin, and of a few dilutions of the preparation itself, into one or two guinea-pigs. The ease, convenience and economy are of less importance than the fact that the standardisation of the preparation is thus based on sound immunological principles.

Further progress was impeded in Germany by the first world war, but in America from 1913 onwards Park's[12,13] energy and enthusiasm, his advocacy of the need for active immunisation, and his demonstration of the practicability and successful use of the T.A. preparations then available, provided a stimulus to workers in other countries and a model on which their efforts could be based.

1921 saw the beginnings of immunisation against diphtheria in England. In April, Glenny, Allen and O'Brien[14] reported on active immunisation and the Schick Test. They outlined the measures which were practicable at that time and described the materials available. Later in the same year Copeman[15] presented an important report on diphtheria to the Ministry of Health and described investigations at the Southmead Infirmary at Bristol; and in December, Copeman, O'Brien, Eagleton and Glenny[16] gave a lucid account of how the Schick Test had been used to detect the susceptibles in a school at Mitcham, how 102 of these had been immunised with T.A., how all but two of these had become Schick-negative, which of them had shown local reaction and the character of those lesions. It is to be regretted that the recommendations of these early workers were not adopted as it is now recognised that many lives would thereby have been saved, much needless suffering avoided, and the conquest of diphtheria expedited.

Park and the early pioneers gave much thought and experiment to find the best mixture of T.A. for the immunisation of children. The balance of toxin and antitoxin had to be precisely adjusted: if the toxin were over-neutralised the mixture was not an effective antigen and if it were under-neutralised it might be unduly toxic and even dangerous. That the right balance had been struck was determined by injecting prescribed quantities into guinea-pigs, and thus the practice of ensuring the safety of prophylactics by tests on animals was begun. These have been developed so that tests for absence of specific and non-specific toxicity, antigenic activity, etc., have been incorporated in the official regulations and national pharmacopœias of many countries. Actually, T.A. was not easy to prepare and, of all the types of prophylactic which have been used, it gave the greatest anxiety. This was due to the fact that it contained toxin and, although this is neutralised with antitoxin and the preparation should be safe, it was a potentially dangerous substance and fatal accidents followed its use in several countries. Viewed in retrospect and in the light of what is now known of the way T.A. was made, the nature of the union of toxin and antitoxin and the circumstances under which they can dissociate, the remarkable thing is, not that there were a few accidents, but that there were not many more. In this country the authorities were so concerned at the possibility of accident that, for many years and until T.A. became obsolete, samples of every batch released for use were tested in the Department of Biological Standards at Hampstead for safety and antigenic activity.

It will be readily understood, therefore, that a milestone of very great importance in the development of diphtheria prophylactics was the discovery that the dangerous toxin could be replaced by harmless toxoid. In 1898 Salkowski[17] observed that diphtheria toxin lost its toxicity when treated with formalin. Later workers have determined the conditions under which toxicity can be removed and immunising efficiency retained: in general it has been found that when incubated at 37°C. with a concentration of 0·5 per cent., or less, of formalin, diphtheria toxin loses its toxicity completely, usually in about four weeks, but it retains its combining power for antitoxin almost unchanged; and Lowenstein[18], Glenny[19,20,21] and Ramon[22,23,24,25] showed that these non-toxic, sterile modifications of toxin——formol toxoid, anatoxin, or F.T. as they are now called—are effective antigens, Lowenstein's experiments relating to tetanus and Glenny's and Ramon's to diphtheria. Formol-toxoid is easy to prepare, it does not contain sensitising horse serum protein, it is quite devoid of specific toxicity and its specific reaction with antitoxin, *in vitro*, provides a ready method for its measurement by flocculation methods. In France it was used on a very large scale for the immunisation of children, and later of soldiers, and its success was such that it was widely used in other European countries and in North America. Its adoption in the United States followed the examination by Park and his colleagues[26] of samples sent to him by O'Brien and his colleagues, and the successful application of toxoids made in his own laboratory[27]:

an important factor in the substitution of F.T. for T.A. in North America in 1924 was the complete safety of the former and the rather frequent reminder that the latter might, on occasion, become toxic.

In this country, apart from its use during several years for the London County Council's immunisation campaign, formol-toxoid was not so extensively used as in other countries and one reason for this was that F.T. was liable to cause unpleasant local reactions, especially in adults: in this country this has always been regarded as a serious defect partly because of the adverse effect on the immunisation campaign. It is possible that this tendency to cause local reactions may have been due to the culture medium employed because formol-toxoid prepared in other countries, on the type of medium favoured by the French workers, appears to have been less objectionable in this respect. Another reason for the limited use of F.T. in England was that whereas, in many other countries these preparations were found to be so safe, so easy to prepare and control and so certain and regular in their behaviour, they were regarded as almost approaching the perfect prophylactic, in this country formol-toxoid has been regarded, not as the last word, but rather as the valuable raw material out of which more perfect antigens could be prepared.

It is interesting to note that it was in this country alone that the attempt was made to render T.A. safe by replacing the toxin by toxoid. Good service had been given by T.A. but it had now become possible to replace it by formol-toxoid, or improve it. Most countries adopted the former course, but in this country an improved and entirely safe type of prophylactic was developed from the original T.A. It was known that under-neutralised mixtures of toxin and antitoxin were excellent antigens, but they were toxic and might be dangerous; by using toxoid, or even toxin which had not been deprived of the last traces of toxicity, under-neutralised mixtures could be prepared, the antitoxin present being more than enough to neutralise the traces of toxin which might remain but insufficient to neutralise all the toxoid. These toxoid-antitoxin mixtures (T.A.M.)[28] were much easier to prepare than T.A. because precise adjustment of the two reagents was not so important, and some toxoid could be left unneutralised; they were widely used and retained their popularity long after more active, and in some ways more satisfactory, antigens became available. One instance of the successful use of T.A.M. was in Birmingham[29], where some 60,000 persons, mainly juvenile, were immunised: the fully detailed and documented record of this city's effort should be studied, for it demonstrated clearly that the disease could be held at bay, many hospital wards could be devoted to other uses and diphtheria brought under control at little cost; and it showed that the benefits which earlier workers had forecast would, indeed, be the reward of the sustained efforts they had called for ten years earlier.

A study of the properties and behaviour of the diphtheria prophylactics available at this time showed that some of them were prone to give

rise to local reactions on injection; and that different types of prophylactic produced their immunising effects in animals, and probably also in man, in different ways. Many observations suggested that the former phenomenon—local reaction—was associated with the non-specific impurities of the prophylactic, some being provided by the broth constituents on which the diphtheria organism was grown, others coming from the products of its metabolism and still others coming from the horse serum which provided the antitoxin of the preparation; and that the latter phenomenon—the mode of action—was associated with the size of the antigenic complex, the rate of its absorption and elimination, the long-continued stimulus which it exerted on the immunising mechanism and, perhaps, the slow dissociation of the combination, or slow release of antigen[30]. Much information was gained concerning both these points by a study of the antigenic properties of the well-washed precipitates derived from neutral, under-neutralised and over-neutralised mixtures of toxin and antitoxin[31,32]. It was shown that nearly the whole of the activity of a mixture is contained in the precipitate which separates from it, that the nitrogen content is remarkably low, that little activity remains in the supernatant fluid, and that the antigenic activity of the floccules, or precipitate, is directly related to the composition of the mixture from which it separates. The sterile emulsions of these toxin-antitoxin floccules is a highly purified form of prophylactic and one of the earliest to be prepared and, being free from non-specific impurities, it seldom gives rise to local reactions on injection. In a later series of experiments[33] the effect of the various impurities, removed during manufacture, on the antigenic activity of the highly purified floccule preparations which remained was determined.

The main results of these investigations were confirmed by Schmidt and Scholtz[34], and from their results and those of Eberhard[35] they considered that these washed toxin-antitoxin precipitates constituted the best antigens available at that time and they were used to a considerable extent in Germany; but, although available, they were not used in England. This was because the original floccule preparations contained toxin and, however firmly bound to antitoxin this may be, it does constitute a potential source of danger. In 1927, however, Glenny and Pope[36] showed that the toxin could with advantage be replaced by toxoid and that the resulting Toxoid-Antitoxin Floccules (T.A.F.) preparation is a highly effective antigen, possessing all the advantages of the similar precipitates prepared with toxin and is completely safe. T.A.F. quickly established itself as an effective antigen (Harries[37], Swyer[38], Freeman[39]) and remains to-day, more than twenty years after its introduction, as one of the antigens of choice in this country. On the large scale it is prepared by allowing the precipitate which separates from under-neutralised mixtures of toxoid and antitoxin to sediment, washing the precipitate several times and finally emulsifying it in saline or other suitable solution in one-tenth, or less, of the volume of the original mixture. T.A.F. is characterised by the infrequency of local or general reactions following its injection, a

quality which makes it particularly suitable for the immunisation of adults, and by its efficacy as indicated by the high Schick conversion rate following injection. The fact that it contains horse serum and that three spaced injections must be given are drawbacks to its use.

The introduction of Alum Precipitated Toxoid (A.P.T.) is due to the work of Glenny[40,41] and his collaborators. There was much evidence to show that increased antigenic activity was associated with the slow absorption and slow elimination of precipitated antigens[42] and the behaviour of alum precipitated toxoid supported this view. The early preparations were used for the immunisation of animals and later Glenny and Barr[41] emphasised the importance of removing the non-specific impurities from preparations intended for use on human beings. To this end Pope and Linggood[43] described the preparation of a culture medium of low nitrogen and controlled iron content which yielded high-grade toxins; the toxoid prepared from these, after purification with charcoal, is precipitated with a predetermined quantity of potash alum and the precipitate is further purified by treatment with disodium phosphate and saline. The antigenic value of the original toxoid is increased from fifty to a hundred fold by converting it into Alum Precipitated Toxoid[44].

Alum Precipitated Toxoid has advantages not possessed by other prophylactics which preceded it. It is a relatively pure substance, free from sensitising horse serum, slowly absorbed from the tissues and is not liable to cause local reactions especially after intramuscular injection; most important of all its advantages is that it is effective when administered in two small spaced doses while other prophylactics require to be administered by three injections. It is very certain in its effects; since 1941 several million persons, mainly juvenile, have received two injections and although it has not been possible to subject them all to the Schick Test, it has been shown in small communities which have been so tested that very high conversion rates followed such injections[45]. Expert opinion is divided as to whether the Schick Test should be applied after a course of inoculations has been given, but more workers would agree that a case for its omission is more easily made out for A.P.T. than for any other type of prophylactic. It is claimed that by the correct use of preparations of A.P.T. of proved efficiency two visits to the clinic will suffice, instead of three, four and sometimes five, which may be required with other forms: this is an administrative convenience the importance of which should not be underrated.

With a view to improving still further the antigenic value of crude formol-toxoid Holt[46], at the Wright-Fleming Institute at St. Mary's Hospital, by the use of semi-synthetic medium in which the iron, salt and nitrogenous constituents were carefully adjusted, prepared high-grade toxins. The optimum conditions for their conversion into toxoid were established and then, by treatment with magnesium and cadmium compounds, followed by fractionation with ammonium sulphate, the large-scale production of purified toxoids of high activity was achieved. An

important development was the successful drying of these toxoids from the frozen state[47] and the optimum conditions for their adsorption on to pure aluminium phosphate. Bousfield[48] has reported very favourably on the clinical results obtained in the field. Tests on animals and human beings show that this latest form of prophylactic—P.T.A.P., as it is called —is a very active antigen and not liable to cause reactions after injection: the effect of the quantity of mineral constituents present in the completed antigen has also been investigated and some evidence has been obtained that the antigenic activity actually increases on storage, even under ordinary room-temperature conditions. A new point of great interest and of useful practical application is that, since large quantities of the highly purified toxoid can be prepared in the dry condition, and that the rest of the antigen is provided by pure inorganic salts added in a simple manner, the opportunity is exceptionally favourable for the preparation of successive batches of P.T.A.P. of uniform potency: tests of six successive batches on groups of 150 children confirmed this expectation[49]. In view of the almost insuperable difficulties involved in the biological assay, by comparative tests in relation to the appropriate standards of the different types of diphtheria prophylactic employed in Great Britain, this possibility of ensuring a continued supply of antigen of proved efficacy is an important advance.

The different types of diphtheria prophylactic which research has provided for field use during the past thirty years form a logical sequence and, although the types used to-day are very different from those available to the early pioneers, each new arrival owes something to its predecessor. In the beginning the danger due to specific toxicity was a source of real anxiety and the factor which most limited progress for many years, but this was removed with the discovery and introduction of toxoid and then the question of local and general reactions claimed more attention. Active immunisation remains, however, a procedure involving the injection of small children, and the inclusion of the Schick Test increases the number of injections and adds other difficulties for the field worker; to any advance which reduces the number of injections must be assigned high merit. Accordingly, the object of research into the materials to be used for active immunisation has resolved itself into the search for a prophylactic which is free from all danger, which does not cause local or general reactions, which is certain in its action, which is effective after a minimum number of injections, which produces a high level of immunity without undue delay, and an immunity which is long-continued and permanent, and not transitory.

There has been steady progress towards the realisation of these aims, but new problems constantly arise. The question of the " injection of recall," or the " boosting dose " is more important than ever. Hitherto, the necessary stimulus to the immunising mechanism established by the injection of prophylactic could be looked for, in part at least, in the exposure of the immunised person to sub-clinical infection; but with the decline in the incidence of the disease this can no longer be counted

upon. For this decline in this country we are indebted to the long-continued, many-sided labours of medical officers of health and others who have not only organised campaigns and acted as the instruments of national policy, but have actually immunised seven millions of our people: but while there are solid grounds for satisfaction, there are none for complacency and the effort must be maintained. In the absence of cases of diphtheria, as a reminder and a threat, it may not be so easy in future to maintain this high rate of primary immunisation, nor to convince the immunised adult that re-injection is necessary for his continued protection and safety. Epidemiologists and immunologists know that the present position of almost assured protection can so deteriorate that, with an inadequately immunised juvenile population and an adult population in which the immunity has declined, an extremely serious situation may develop; the reason for this may not be easy to explain to the layman, nor the reason why he cannot acquire protection against diphtheria as easily as when threatened with smallpox. Much thought, laboratory experiment and field trial will no doubt be given in order to reach a decision as to the best type of prophylactic to be used, the dosage, the route and the time for the re-injection of our young people all of whom, it is hoped, will have been fully inoculated before going to school. It does not necessarily follow that the type of antigen which succeeded so well in laying down a basal immunity is necessarily the best to use for the boosting dose or injection of recall, but there is now almost an embarrassment of choice of highly purified prophylactics available. The present indications are that it may be necessary to test each candidate for reinjection by the application of the Schick Test or some modified form of it, and to proceed in accordance with the result of that test. It will be interesting to see whether the increasing use of highly purified antigens affects the reaction of the individuals, and populations, to subsequent injections in later life.

REFERENCES

1. Smith, *J. med. Res.*, 1907, **16,** 359.
2. Smith, *J. exp. Med.*, 1909, **11,** 241.
3. Park and Famulener, *Proc. Soc. exp. Biol., N.Y.*, 1908-9, **6,** 98.
4. Schick, *Munch. med. Wschr.*, 1913, **60,** 2608.
5. Römer, *Z. Immun. Forsch.*, 1909 **3,** 208.
6. Behring, *Dtsch. med. Wschr.*, 1913, **39,** 873.
7. Behring, *Berl. klin. Wschr.*, 1913, **51,** 917.
8. Trevan, *Proc. roy. Soc.*, 1927, **101B,** 483.
9. Glenny, *Lancet*, 1922, **202,** 228.
10. Kellogg, *J. Amer. med. Ass.*, 1922, **78,** 1782.
11. Glenny and Waddington, *J. Path. Bact.*, 1929, **32,** 275.
12. Park, *Amer. J. Obstet. Gynec.*, 1913, **68,** 1213.
13. Park, *J. Amer. med. Ass.*, 1922, **79,** 1584.
14. Glenny, Allen and O'Brien, *Lancet*, 1921, **200,** 1236.
15. Copeman, *Report on Diphtheria, Ministry of Health*, 1921, No. 10.
16. Copeman, O'Brien, Eagleton and Glenny, *Brit. J. exp. Path.*, 1922, **3,** 42.
17. Salkowski, *Berl. klin. Wschr.*, 1898, **35,** 545.
18. Lowenstein, *Z. Hyg. InfectKr.*, 1909, **62,** 491.
19. Glenny and Sudmerson, *J. Hyg., Camb.*, 1921, **20,** 176.
20. Glenny and Hopkins, *Brit. J. exp. Path.*, 1923, **4,** 283.

21. Glenny, Hopkins and Pope, *J. Path. Bact.*, 1924, **27,** 261.
22. Ramon, *C.R. Acad. Sci., Paris,* 1923, **177,** 1338.
23. Ramon, *Ann. Inst. Pasteur,* 1923, **37,** 1001.
24. Ramon, *ibid.,* 1924, **38,** 1.
25. Ramon, *ibid.,* 1925, **39,** 1.
26. Park, Banzhaf and Zingher, *N.Y. St. J. Med.,* 1924, **24,** 49.
27. Park, Banzhaf, Zingher and Schroeder, *Amer. J. publ. Hlth.,* 1924, **14,** 1047.
28. Glenny, Pope, Waddington and Wallace, *J. Path. Bact.,* 1925, **28,** 333.
29. Burn and Fellowes, *Lancet,* 1934, **237,** 1181.
30. Glenny, *J. Hyg., Camb.,* 1925, **24,** 301.
31. Hartley, *Brit. J. exp. Path.,* 1925, **6,** 112
32. Hartley, *ibid.,* 1926, **7,** 55.
33. Hartley, *ibid.,* 1935, **16,** 460.
34. Schmidt and Scholz, *Arch. Hyg. Berl.,* 1925, **96,** 251.
35. Eberhard, *Z. Hyg. InfectKr.,* 1926, **105,** 614.
36. Glenny and Pope, *J. Path. Bact.,* 1927, **30,** 587.
37. Harries, *Lancet,* 1930, **218,** 802.
38. Swyer, *ibid.,* 1931, **220,** 632.
39. Freeman, *Brit. med. J.,* 1942, **2,** 123.
40. Glenny, Pope, Waddington and Wallace, *J. Path. Bact.,* 1926, **29,** 31.
41. Pope and Linggood, *Brit. J. exp. Path.,* 1939, **20,** 297.
42. Glenny, *Brit. med. J.,* 1930, **2,** 244.
43. Pope and Linggood, *Brit. J. exp. Path.,* 1939, **20,** 297.
44. Barr, Glenny, Pope and Linggood, *Lancet,* 1941, **241,** 301.
45. Fulton, Taylor, Moore, Wells and Wilson, *Brit. med. J.,* 1942, **1,** 315, 349.
46. Holt, *Brit. J. exp. Path.,* 1948, **29,** 335.
47. Holt, *Lancet,* 1947, **252,** 282.
48. Bousfield, *ibid.,* 1947, **252,** 286.
49. Bousfield and Duke, *Med. Officer,* 1948, **80,** 145.