THE PREPARATION, TESTING AND STANDARDIZATION OF TYPHOID VACCINE

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THE PREPARATION OF ALCOHOL-KILLED AND ALCOHOL-PRESERVED TYPHOID VACCINE

The introduction, ten years ago, of a new type of typhoid-paratyphoid vaccine, killed and preserved with alcohol (Felix, 1941; Felix, Rainsford & Stokes, 1941), has had a stimulating effect on the experimental study of the methods of making T.A.B. vaccine and testing its potency. The time-honoured original T.A.B. vaccine, killed by heat and preserved with phenol (Wright & Semple, 1897; Pfeiffer & Kolle, 1896), had held the field for over forty years. Although a great number of different modifications of the original method had been described during the intervening years, nearly all of them had been abandoned, since none was found to be superior to the simple method of heat-killing and phenol-preservation. At the outbreak of the war of 1939–45 two other types of typhoid vaccine had their local adherents, namely, vaccine killed by formalin (in Italy) and Grasset's (1939) 'endotoxoid' vaccine (in South Africa), but neither of the two seriously competed with ordinary T.A.B. vaccine.

The case for changing the original method of making typhoid vaccine was based on two well-established facts: first, that the Vi antigen and its corresponding antibody are of outstanding importance in immunity to experimental infection with the typhoid bacillus; secondly, that both heating and treatment with phenol cause considerable damage to the Vi antigen (Felix & Pitt, 1934; Felix & Bhatnagar, 1935). As soon as it was found that alcohol-killed typhoid bacilli stimulated in the rabbit abundant Vi antibody of full protecting and opsonizing power

(Felix & Pitt, 1936; Felix & Petrie, 1938), attempts were made to use alcohol in the preparation of typhoid vaccine. These attempts finally resulted in the use of alcohol not only for the sterilization of the vaccine but also for its preservation (Felix, 1941). The advantages of the alcohol-treated vaccine, both in regard to the Vi-antibody response and the degree of systemic reactions produced, soon led to its adoption in this country. The new type of vaccine was first used in 1941 by the Emergency Public Health Laboratory Service (Medical Research Council) for inoculation of civilians; it was adopted by the Royal Air Force in 1943 and by the Army in 1944.

The technique employed for the preparation of the alcohol-killed and alcohol-preserved vaccine is very simple, and has remained essentially unchanged throughout the years. The only alteration in the method, as originally described, was that 25% alcohol was used instead of 22·5%. This change was made almost from the start, when comparative tests showed that 25% alcohol was markedly superior to 22·5% alcohol in its power to destroy bacteria that may contaminate the vaccine (Cruickshank, Hobbs, McFarlan & Maier, 1942). At the same time, it was established that the higher concentration of alcohol in the vaccine did not give rise to stronger local or constitutional reactions. A comparison of reactions to alcoholized and phenolized T.A.B.C. vaccines prepared at the Lister Institute from the same four strains of typhoid and paratyphoid bacilli was made by the Emergency Public Health Laboratory Service (1942). In another trial the alcoholized T.A.B.C. vaccine was compared with phenolized T.A.B. vaccine made from different strains at the Army Vaccine Department. Air Commodore T. McClurkin, Director of Hygiene, Air Ministry, who arranged the trial in R.A.F. personnel in 1942, summarized his observations in the following table (Table 1).

Table 1. *Comparative trial of alcoholized T.A.B.C. and phenolized T.A.B. vaccines in R.A.F. personnel* (*Air-Commodore McClurkin*, 1942, *personal communication*)

Vaccine used	No. inoculated	No. sick following inoculation		Average number of days sick	Total number of days sick
Heat-killed phenolized T.A.B. vaccine (Army Vaccine Department)	2000	1st dose	8	4·5	116
		2nd dose	20	4	
Alcoholized T.A.B.C. vaccine (Lister Institute)	2000	1st dose	5	3·4	34
		2nd dose	5	3·4	

During the past ten years a number of workers have compared the alcohol-treated vaccine and the heat-killed phenolized product for their immunizing potency, and for the degree of local and constitutional reaction following their administration (Rainsford, 1942; Climie, 1942; Gohar & Elian, 1942; Griffits, 1944; Drysdale, 1947; Amaral & Lacerda, 1947). Their findings are in general agreement with those of Felix (1941) and Felix *et al.* (1941). In addition, various chemicals have been compared with ethyl alcohol for their suitability as killing and preserving agents. To mention only a few of the suggested substances and

procedures: killing with sodium merthiolate and preserving with 32% saline solution, or desiccation after treatment with acetone (Rainsford, 1942); killing and preserving with ether (Gohar & Elian, 1942); sterilizing with merthiolate and preserving with molar (34%) sucrose solution (Loureiro, 1946). In each instance the examination of these differently prepared typhoid vaccines was based mainly, if not exclusively, on a comparison of the properties of their Vi-antigen component. Although it was claimed that some of these various treatments preserved the Vi antigen even better than did 25% alcohol, none of the suggested procedures led to the development of a method suitable for large-scale preparation of typhoid vaccine.

It was stated in the original description of the method that the alcohol-treated suspensions of typhoid and paratyphoid bacilli have an opacity considerably less than that of suspensions equivalent in numbers but killed by heat and preserved with phenol. The difference in opacity is about 10–20%, and is accounted for partly by the destruction of the flagella and partly by some alteration in the physical condition of the surface of the bacteria. It is, therefore, recommended to estimate the bacterial content of the saline suspensions of living bacilli immediately they have been washed off the agar medium (Roux bottles). During the treatment with 75% alcohol there need be no loss in bacteria, and the final suspensions in 25% alcohol are made up to the volume calculated from the original estimation of the living saline suspensions.

VACCINE STRAINS

The methods used for the selection of the most suitable vaccine strains and the preservation and maintenance of the cultures have also remained unchanged. The ideal to be aimed at is, of course, that such strains should at all times develop the O and Vi antigens in maximum amount when grown on plain nutrient agar (Felix, 1941). The three strains that were originally chosen for their outstanding ability to fulfil this condition are: *Salmonella typhi* strain Ty2 (Weil & Felix, 1920); *Salm. paratyphi A* strain HA6 (Felix & Olitzki, 1926); *Salm. paratpyhi B* strain HB3 (Felix & Pitt, 1936). These three strains are still widely used as vaccine strains in this and many other countries.

The technical details to be observed in the quantitative estimation of the Vi- and O-antigen content of the typhoid vaccine strain Ty2 have been described recently (Felix & Pitt, 1951). Those relating to the two paratyphoid vaccine strains HA6 and HB3 will be discussed in a subsequent paper (Felix & Pitt, to be published). It may, however, be useful briefly to mention here—in view of the persistent claim by Kauffmann (1941*a*, 1947) that *Salm. paratyphi A* and *Salm. paratyphi B* do not possess Vi antigens similar to that of *Salm. typhi*—that the selection of the vaccine strains HA6 and HB3 has been made by precisely the same criteria as those applied to the virulent typhoid Vi+O strain Ty2. The paratyphoid-B strain HB3 is the strain labelled no. 3 in Table III on page 84 in the paper by Felix & Pitt (1936), which was found to contain the BVi antigen in maximum quantity. The paratyphoid-A strain HA6 was selected at the same time by the same method.

Like strain Ty 2, the two paratyphoid strains HA 6 and HB 3 have retained their respective antigenic properties in a remarkably constant manner throughout the years. It is well known that it is particularly difficult to maintain strains of *Salm. paratyphi A* at the level of mouse virulence they exhibit when freshly isolated. Nevertheless, Callender & Luippold, who adopted the strain HA 6 as the paratyphoid-A vaccine strain for the United States Army vaccine, stated that it 'is easily the most virulent para A organism that we have found' and 'immunologically superior to all other strains with which we have worked' (Callender & Luippold, 1943, p. 320). Dr J. H. Mason, of the South African Institute for Medical Research, Johannesburg, who adopted the two strains HA 6 and HB 3 as vaccine strains in 1947, also found both of them to be much more virulent for mice than any of the strains that had been in current use at the Johannesburg Institute (Mason, 1947, personal communication).

It is often suggested, mostly by clinicians and epidemiologists, that it may be preferable in the preparation of T.A.B. vaccine to employ a mixture of several strains of typhoid and paratyphoid bacilli, including recently isolated local strains. There is no evidence available to support this view. It has been emphasized in earlier work that freshly isolated strains of typhoid or paratyphoid bacilli are not necessarily the best source of the O and Vi antigens that are essential in the make-up of a potent vaccine. Though many strains at the time of isolation are highly virulent and possess their specific Vi antigen in maximum amount, only few have the ability to maintain it at a high level when subcultured on artificial media.

The question of a difference in immunogenic properties associated with different typhoid Vi-phage types has also been raised. It has been mentioned in previous papers (Felix, 1941, 1944) that this point was carefully investigated immediately after the publication by Craigie & Yen (1938) of their important work on typhoid Vi-phage types. No serological differences could be detected in the Vi antigen of typhoid strains belonging to different phage types. The experiments included cross-agglutination and cross-absorption tests, and also phagocytosis and passive-protection tests in mice (Felix & Pitt, unpublished). It appears, therefore, safe to continue employing a single Vi + O strain of the typhoid bacillus for making typhoid vaccine. By analogy, the same may be assumed to apply to the various Vi-phage types of *Salm. paratyphi A* and *B*.

METHODS OF ASSAYING THE PROTECTIVE VALUE OF TYPHOID VACCINE

Active-protection tests in mice have been the most widely used method of assessing the protective value of typhoid vaccines, since Grinnell (1932) first showed that the mouse is a suitable experimental animal for virulence and protection tests with the typhoid bacillus. At a very early stage, however, it was found that the active-immunity experiments in mice do not disclose those important differences in the antigenic value of various preparations of O + Vi bacilli which are so clearly demonstrated by passive-protection experiments in mice or even by *in vitro* laboratory tests.

The presence of Vi antigen in typhoid vaccine is known to be essential for the protection of mice against virulent typhoid bacilli. It is also known that the

Vi antigen is a peculiarly labile substance. Sterilization by phenol or heating to 58° C. so lowers the efficacy of typhoid vaccine that its power of stimulating a Vi-antibody response in the rabbit, the horse or in man is almost completely suppressed, or at least greatly reduced. Sterilization by formalin so alters the Vi antigen that the resulting antibody exhibits a peculiar 'functional deficiency', i.e. it acts effectively in the agglutination reaction, but its power of promoting phagocytosis and of protecting mice passively against infection with virulent O + Vi bacilli is greatly reduced. On the other hand, alcohol-treated vaccine stimulates in the rabbit, the horse and man the production of a Vi antibody which possesses the full functional efficacy of the antibody induced by immunization with the 'natural' Vi antigen contained in living bacilli (Felix & Pitt, 1934, 1935, 1936; Felix, Bhatnagar & Pitt, 1934; Felix & Bhatnagar, 1935).

When, however, vaccines prepared from the same culture and sterilized by a variety of methods, including those just mentioned, were compared in active-protection tests in mice, no significant difference in their protective value could be detected. Schütze (1936) compared ten different methods of sterilization, and found all of them to be of approximately equal value in the active immunization of mice.

This major discrepancy in the behaviour of typhoid vaccines in active- and passive-protection tests in the mouse became obvious at a very early stage. It was, indeed, an experiment on active immunization of mice with heat-killed typhoid bacilli that indicated for the first time 'that the factor responsible for inagglutinability and virulence is an antigen and that the corresponding antibody is a powerful protecting agent' (Felix & Pitt, 1934, p. 187). It was the recognition of this fact that led to the discovery of the Vi antigen. Nevertheless, the facts underlying the observed differences in the results of the two sets of experiments are often referred to in a misleading manner. For instance, Batson (1949, p. 23), in a review of methods of production of typhoid vaccine, stated: 'Both heat and phenol reportedly destroy the Vi antigen' and quoted as authority for this statement the papers by Felix & Pitt (1934) and Felix & Bhatnagar (1935). Spitznagel & Trainer (1949, p. 234), in another paper on typhoid vaccine published from the United States Army Medical Department [Washington], referred to formalin 'which has little effect on the Vi antigen' and to heat 'which destroys the Vi antigen'. These statements, however, have no foundation in fact.

Felix & Bhatnagar (1935) found that sera from rabbits immunized with phenolized vaccines did not contain demonstrable Vi antibody, and consequently had no protective action in passive immunization of mice. This finding, the authors stated, demonstrated 'the extreme susceptibility to phenol of the Vi antigen of *B. typhosus*' (Felix & Bhatnagar, 1935, p. 439). In later experiments it was shown that the inactivation by phenol was due to a reversible reaction between phenol and the Vi antigen. When the phenol was removed from the suspension and replaced by fresh saline, such phenol-treated vaccines invariably stimulated the production of circulating Vi antibody in the rabbit (Felix & Petrie, 1938). It is therefore incorrect to state that phenol or heat 'destroy' the Vi antigen. Each of these treatments, however, has a most damaging effect on the Vi-antibody-producing power of the

typhoid bacillus. There were certainly good reasons for the general recognition of the Vi antigen as the heat-labile somatic antigen of this organism.

When assessing the protective value of typhoid vaccine, some workers consider it unnecessary to demonstrate circulating antibodies in a suitable animal or in man, or to test these antibodies for their efficacy in passive-immunity experiments. This view is evidently based on a wrong interpretation of what is known about the mechanism of active antibacterial immunity. It is, of course, recognized that active immunity depends mainly on the increased power of the tissues to elaborate specific antibodies rapidly when infection occurs, and the amount of circulating antibodies found in the individual animal or man is not a reliable measure of this power. It is, however, equally well established that both the increased cellular reactivity and the circulating antibody owe their existence to the specific stimulus of the same substance—namely, the specific bacterial antigen. Consequently, it is of fundamental importance to preserve the antigen in that form which most effectively stimulates both the cellular reactivity and the production of circulating antibody. Recovery from the disease is known to produce both these signs of immunity response and to confer the highest degree of immunity to typhoid infection that man can acquire.

It has been shown recently (Findlay, 1951; Felix & Anderson, 1951*b*) that properly conducted virulence tests in mice do, in fact, reflect the relative virulence for man of typhoid strains isolated from different outbreaks. Nevertheless, it is important to remember that the typhoid bacillus is not a natural pathogen of the mouse, and this may be the reason for the discrepancy in the results of active- and passive-protection experiments in this animal. Whatever the explanation of this phenomenon, it is clear that it is illogical to accept two differently prepared vaccines as being of equal immunizing value, when the one readily stimulates formation of circulating Vi antibody in the rabbit or man and the other does not, or when one of the vaccines induces formation of an antibody of high protective power and the other a modified antibody possessing only a fraction of this power. Since the customary active-immunity test in the mouse does not disclose these important differences in the antigenic properties of different preparations of typhoid antigens, it cannot be considered to be a sufficiently sensitive method of testing typhoid vaccines, and certainly should not be accepted as the sole test.

Phagocytosis tests also indicated at an early stage that mouse-protection experiments are not the most sensitive tools in the elucidation of problems of typhoid immunity. Passive-protection experiments in mice undoubtedly are more sensitive than active-immunity tests, but phagocytosis experiments excel both types of mouse test. Experiments on passive immunization of mice against infection with an O-inagglutinable virulent O+Vi strain did not indicate any increase in the protective action of the Vi antibody attributable to the activity of the accompanying O antibodies. On the other hand, phagocytosis experiments clearly demonstrated the summation of the sensitizing effects of each of the two antibodies, though the powerful activity of the Vi antibody tended to overshadow the feeble action of the O antibody (Felix & Bhatnagar, 1935).

Because of these well-established facts it was concluded (Felix, 1941) that all the

available methods should be employed in order to detect how far the immunizing properties of differently prepared typhoid vaccines have been preserved or impaired. These methods are:

(1) Active immunization of mice.

(2) Immunization of rabbits with a view to establishing:

  (*a*) whether circulating Vi and O antibodies are readily elaborated;

  (*b*) whether the Vi antibody produced possesses full 'functional efficacy' in passive-protection tests in mice.

(3) Tests of the Vi and O antibody responses in man.

Active immunization of mice is the least sensitive test. A vaccine that has been found to be of low protective value in this test should be condemned, and need not be further examined. The sensitivity of the test can be somewhat increased by employing a series of graded doses of the immunizing antigens and of the challenge organism. This procedure, however, cannot raise the sensitivity of the active-protection test to a level comparable with that of the tests listed under methods (2) and (3).

The alcohol-killed and alcohol-preserved vaccine was adopted when it was found that its performance in these severe tests in the mouse, the rabbit and man was better than that of heat-killed phenolized vaccine.

SOURCES OF ERROR IN MOUSE-PROTECTION TESTS

In addition to the serious discrepancies in the results of active- and passive-immunity experiments in mice that have been discussed in the preceding section, there are a number of factors that have a far-reaching effect on the outcome of mouse-protection tests with the typhoid bacillus.

(*a*) *The degree of mouse virulence of the test culture*

The main interest of the early work on the Vi and O antigens lay in the demonstration that the two played quite different roles in the pathogenic action of the typhoid bacillus, the O antigen being mainly responsible for toxicity and the Vi antigen for protecting the O antigen, and thereby the whole bacterial cell, against the action of the natural or immune O antibody. Additional evidence in support of this view has been recorded in a preceding paper of this series (Felix & Pitt, 1951). With regard to the corresponding two antibodies, it is known that the Vi antibody confers protection against infection with virulent O + Vi strains by suppressing the multiplication of the organisms, whereas the O antibody is chiefly responsible for effecting the neutralization of the endotoxin of the typhoid bacillus. This coherent picture of the pathogenic and immunogenic processes fits well the facts established in mouse-protection tests and in phagocytic and bactericidal experiments *in vitro*.

Great emphasis was laid in earlier work on the fact that the sharp distinction between the activities of the Vi and the O antibody in mouse-protection tests is revealed only when the strains used for the challenge dose possess maximum virulence, are particularly rich in the Vi antigen, and show the highest degree of

resistance to the O antibody (Felix, 1938). Most laboratory strains, however, contain only a moderate amount of the Vi antigen and are, therefore, less O resistant. With such strains a powerful sensitizing effect of the O antibody is readily demonstrated in bactericidal or phagocytic experiments with rabbit or human serum. Pure O antisera, containing no Vi antibody, are therefore capable of exerting a strong protective action in the passive-immunity test in the mouse when such cultures are employed for the challenge dose. If, however, the animals receive a test dose prepared from a fully O-resistant culture, the sensitizing action of the O antibody is feeble and is masked by the much more powerful activity of the Vi antibody. Under these conditions of the experiment, and under these conditions alone, the contribution by the O antibody towards the sum total of protective action of a Vi + O antiserum is almost negligible. This accounts for the close correlation between the mouse-protective value and the Vi-agglutinin titre of the serum, irrespective of the presence in the serum of O antibody even of a very high titre.

These facts made it possible to suggest the mouse-protection test with highly virulent living bacilli as a means of assaying the Vi-antibody content of therapeutic anti-typhoid serum. On the other hand, a mouse test with killed typhoid bacilli as the challenge dose serves for the estimation of the endotoxin-neutralizing power of the serum, which runs parallel with its O-agglutinin titre. The technique of the two tests has been described in detail and a 'Provisional Standard Anti-typhoid Serum' containing both Vi and O antibodies has been suggested at the same time (Felix, 1938). This dried serum preparation has been recommended provisionally as standard by the Health Organization of the League of Nations (Report of Permanent Commission on Biological Standardization, 1938) and is held at the Department of Biological Standards, National Institute for Medical Research, London.

The outstanding importance of the Vi antibody in protection against highly virulent O + Vi strains of *Salm. typhi* has been established in active-immunity experiments as firmly as in passive-protection tests. Neither a pure O-antigen vaccine nor a pure O antiserum is capable of protecting the mouse when a strain of maximum virulence is employed as the challenge organism, whereas a pure Vi-antigen vaccine or a pure Vi antiserum exerts a powerful protective action.

From experience of the strain Ty 2 during the past seventeen years it is known that the approximate minimal lethal dose (M.L.D.) of the test cultures of this strain is usually 80×10^6 organisms, when the standard technique is employed. So long as the approximate M.L.D. is not greater than 100×10^6 organisms the results obtained in comparative mouse-protection tests are consistent and uniform. At this level of virulence the culture is fully resistant to the O antibody, and the degree of protection established in the tests is due almost entirely to the action of the Vi antibody. If the culture falls below this level of virulence, that is to say, when a dose of 100×10^6 organisms kills less than nine out of ten mice, the bacilli are no longer fully O resistant and the degree of protection determined in the test is no longer a measure of the activity of the Vi antibody, but is partly due to the antibacterial action of the accompanying O antibody.

The other laboratory strains that have been extensively employed in mouse experiments with the typhoid bacillus never showed the high degree of virulence exhibited by strain Ty 2. Perry, Findlay & Bensted (1933), who 'rejuvenated' the Rawlings strain by passage through the mouse, found it necessary to recommend its preservation in mouse spleen dried *in vacuo* and stored in sealed ampoules (Perry, Findlay & Bensted, 1934). The 'minimal (average) lethal dose' of this strain is stated by Topley, Raistrick, Wilson, Stacey, Challinor & Clark (1937) to have been 'rather less than 100 million bacilli'; the corresponding figure for the strain Ty 2 is about 40 million bacilli. The challenge dose of 500 million organisms of the rejuvenated Rawlings strain, which Topley *et al.* (1937) used throughout their experiments, killed as a rule only 90% of the normal control mice; when the strain Ty 2 is employed, there is rarely a survivor amongst the control mice inoculated with half that dose. Topley *et al.* (1937) showed that killed O bacilli and the purified O-antigen fraction induced a very definite protective action in active- and passive-immunity experiments with the rejuvenated Rawlings strain. This is obviously due to the fact that this strain, which has played so honourable a part in the history of antityphoid inoculation, is less virulent and less O resistant than the strain Ty 2.

The strain '58', isolated in 1934 from a chronic carrier known as the 'Panama Carrier' and used for the preparation of United States Army vaccine since 1936 (Siler, Dunham, Longfellow & Luippold, 1941), is even less virulent than is the rejuvenated Rawlings strain, and it has been so from the outset. As early as 1935 the average lethal dose (A.L.D.) of strain 58 was found to be 100 million organisms, whereas that of the rejuvenated Rawlings strain, which was examined by the American workers at the same time, was stated to be 50 million organisms (Siler *et al.*, 1941, p. 62). In more recent papers, the virulence of strain 58 is put at an even lower level. Batson, Landy & Abrams (1949) found that a dose of 270 million organisms killed only six out of twenty mice. Batson, Landy & Brown (1950*a*) give the approximate L.D. 50 value of this strain as 220 million organisms. The American workers, nevertheless, refer to strain 58 as being 'highly virulent'. Disregarding what had been established by earlier workers, Batson, Landy & Brown went even further and selected as the challenge organism in active-immunity experiments in the mouse a typhoid strain ('63') which, in the authors' own words, 'is markedly less virulent than is strain 58' (Batson, Landy & Brown, 1950*b*, p. 241). It is not surprising, therefore, that these experiments did not reveal any relationship between the Vi antigen and the immunizing potency of typhoid vaccine.

It may be asked whether it is justifiable to conclude from the available experimental evidence that the Vi antibody is of primary importance in the defence of man against infection with the typhoid bacillus. The information accumulated during the past fifteen years clearly indicates that practically all strains freshly isolated from the blood of typhoid patients contain the Vi antigen. Earlier reports, including that by Felix, Krikorian & Reitler (1935), on the isolation from the blood of typhoid patients of strains devoid of the Vi antigen were obviously due to inadequate methods of growing and testing the cultures. The reports by different

workers differ only with regard to the relative frequency, among freshly isolated blood cultures, of those Vi + O strains that are fully O resistant and those that are partly O agglutinable (i.e. intermediate Vi + O forms). Cultures isolated from chronic carriers or temporary excreters are, as a rule, also virulent Vi + O forms, although they may be converted into the Vi-negative form by the action of anti-Vi bacteriophage (Craigie & Brandon, 1936). How readily O inagglutinability is lost on subculture has been shown by Felix *et al.* (1934) and by Craigie & Brandon (1936). It may, therefore, be concluded that it is the O-resistant highly virulent O + Vi form that is of significance in human infections with *Salm. typhi*.

It is, of course, not claimed that the strain Ty 2 holds a unique position. Numerous workers in various parts of the world have described strains of *Salm. typhi* which, at the time of their isolation, possessed maximal O inagglutinability and maximal virulence for the mouse. The strain Ty 2 is superior to other strains in maintaining these characters almost indefinitely when grown on plain agar, the medium used routinely in the manufacture of typhoid vaccine. Since its isolation in 1918, the strain has been maintained without a single passage through an animal or subculture on the more complicated media, and this may have contributed to its remarkable stability (Felix, 1938; Felix & Pitt, 1951).

It has been known for a long time that the results of comparative protection tests with the typhoid bacillus are decidedly influenced by the degree of virulence of the test organism. Mice from the same batch of actively immunized animals were found to be better protected against a highly virulent strain than against a relatively avirulent strain (see Table V of Felix & Pitt, 1934). This paradoxical result at first appeared difficult to interpret, but it was readily explained when it was recognized that the course of typhoid infection and the defence against it depend on the interaction between two pairs of antigens and antibodies, each of which plays a different part in the process.

Similar mechanisms are undoubtedly at work in other pathogenic organisms. A most interesting analogy is provided by the recent observations of Habel & Wright (1948) on the factors influencing the mouse-potency test for rabies vaccine. These workers found that 'a given vaccine could be demonstrated to be of very low or of very high potency depending upon whether a highly invasive virus or one of low invasiveness was used as challenge material'. Since all the strains used in the experiments of Habel & Wright were substrains of the original Paris (Pasteur) fixed rabies virus, heterogeneity of antigens could not be suspected. In the present writer's view the important finding by Habel & Wright clearly indicates that at least two pairs of antigens and antibodies are concerned in rabies infection and immunity, in much the same way as in the case of infection with, and immunity to, the typhoid bacillus.

(*b*) *The mucin technique*

Another important source of error in mouse-protection tests with the typhoid bacillus, operating in both active- and passive-immunity experiments, is the use of the mucin technique. Whatever its merits or demerits in the study of other bacterial infections, it is safe to say that the widespread use of the mucin technique has had a most calamitous effect on experimental work with the typhoid bacillus.

Although the exact mechanism of the action of mucin is still unknown, it is generally agreed that it acts on both the experimental animal and the infecting bacteria. Ørskov (1940) showed that mucin blocks the migration of phagocytes into the abdominal cavity and also inhibits the bactericidal action which the peritoneal fluid of the normal mouse exerts on Shiga's bacillus. Tunnicliff (1940) found that mucin-coated staphylococci are not phagocytosed. Pacheco & Noronha (1940) reported that mucin *in vitro* inhibited the bactericidal action of cholera antiserum on *Vibrio cholerae*. Thus the normal defence mechanism is put out of action within the area reached by the mucin, and the injected bacteria propagate in the peritoneum as freely as they would in the best culture medium. The small number of bacteria injected with mucin have soon multiplied sufficiently to reach the number that must be injected without mucin in order to produce a lethal effect in the mouse. It does not appear, therefore, to be appropriate to speak of the effect of mucin as 'enhancement of virulence', as many authors do.

In the particular case of the typhoid bacillus this effect of mucin was first reported by Rake (1935), and confirmed by Siler (1936) and Buttle, Parish, McLeod & Stephenson (1937). The virulent O + Vi form and the relatively avirulent 'pure' O and 'pure' Vi variants are influenced by the presence of mucin in the same degree, i.e. the number of organisms representing a lethal dose for the mouse is in each instance reduced 1000- to 10,000-fold (Henderson & Morgan, 1938; Henderson, 1939). The relative mouse virulence of the three different variants, which has been discussed in a preceding paper (Felix & Pitt, 1951), is approximately the same whether the injections are made with mucin or without it.

It was, however, soon found that the presence of mucin in the challenge dose produced a change in behaviour of the O-resistant virulent Vi + O strain, namely, loss of its O resistance. Henderson & Morgan (1938), Henderson (1939), Boivin (1939) and Boivin & Mesrobeanu (1938) made extensive use of the mucin technique in active- and passive-immunity experiments with the strain Ty 2. They found that, under these particular conditions, the strain Ty 2 was fully susceptible to the action of the O antibody. It is known from the work discussed in the preceding sections of this paper that neither a pure O-antigen vaccine nor a pure O antiserum is capable of protecting mice effectively when the tests are conducted in the absence of mucin. In the experiments of Henderson (1939) this was again confirmed when the challenge doses of strain Ty 2 were made up in parallel with mucin and without it, and were tested side by side.

There appears to be no reason for assuming, as many workers have done or still do, that the mucin technique affords an improvement in experimentation with the typhoid bacillus. It is true, this method does make the typhoid bacillus appear to be more 'virulent', because the natural resistance of the mouse is lowered. But the condition following the intraperitoneal injection of the small number of organisms, together with mucin, does not resemble the natural disease in man more closely than does the infection initiated in the mouse by the larger dose of bacilli injected without mucin. The position, of course, would be quite different if a method were found that would render the mouse susceptible to typhoid infection by mouth. This, however, cannot be achieved by the use of mucin as an adjuvant.

It is obvious that the mucin technique introduces additional unknown factors influencing both the infected animal and the bacterium, and thus renders the mouse test still more dissimilar to the natural infection in man.

The position may be summarized as follows:

(1) In mouse-protection tests with a fully virulent O + Vi strain, carried out according to the mucin technique, pure O-antigen vaccine or pure O antiserum is as effective as Vi-antigen vaccine or Vi antiserum.

(2) In mouse-protection tests carried out without mucin the effect of pure O-antigen vaccine or pure O antiserum is almost negligible, whereas that of pure Vi-antigen vaccine or pure Vi antiserum is all-important.

(3) In phagocytic tests with a fully virulent O + Vi strain the Vi antibody by far excels the O antibody in sensitizing activity.

There is thus full agreement between the results of phagocytic experiments and mouse-protection tests carried out without mucin, but a major discrepancy is introduced when the mucin technique is employed. The importance of phagocytosis in the mechanism of antibacterial immunity is too well known to call for further emphasis. In view of these firmly established facts it is not unreasonable to suggest that the mucin technique be abandoned in experimental work on the prophylaxis and treatment of typhoid fever, including the assay of typhoid vaccine.

(*c*) *The route of administration of the immunizing injections and of the challenge dose*

The third pitfall operative in mouse-protection tests is the non-specific rise in natural resistance that follows the intraperitoneal injection of various substances, such as broth, killed vaccines, etc. This phenomenon has been known since the early days of the bacteriological era, when it was first observed in connexion with Pfeiffer's classical experiments with *V. cholerae* (Pfeiffer & Issaeff, 1894; Sobernheim, 1895). It is of considerable importance in all protection experiments in which the challenge dose is administered by the intraperitoneal route.

To avoid the effect of non-specifically increased intraperitoneal resistance the present writer has invariably employed different routes for the immunizing and the challenge doses. In active-immunity experiments vaccines were always injected subcutaneously, and the challenge dose was given intraperitoneally after an interval of about 3 weeks. In passive-immunity tests the sera were given intramuscularly and the intraperitoneal challenge dose followed 2 days later (Felix & Pitt, 1934; Felix, 1938). Unfortunately, many workers engaged in experiments on typhoid immunity have used, and continue to use, the faulty method of injecting both the immunizing and the challenge doses by the intraperitoneal route.

Ørskov & Kauffmann (1936), Philipson (1937) and Ørskov (1940) examined very carefully the degree and duration of this kind of non-specific immunity. For instance, one intraperitoneal dose of a vaccine of *Salm. paratyphi B* induced in mice a high degree of immunity to intraperitoneal challenge with *Shigella shigae*, and this non-specific effect was demonstrable for 9 days. When two doses of vaccine were given at a 9-day interval, the immunity lasted up to 21 days (Philipson, 1937).

It is clear from these well-established facts that much of the work on typhoid vaccines is invalidated because due attention has not been paid to this important pitfall.

SOURCE OF ERROR IN THE RABBIT TEST

The estimation of circulating Vi and O antibodies in rabbits immunized with different typhoid vaccines has been practised for many years, and proved to be a reliable and sensitive method of detecting any damage that may have been done to the Vi antigen in the course of preparation of the vaccine. A particular source of error in this test was, however, encountered some years ago.

The standard procedure in the test is, of course, to examine the serum of the normal rabbits before they are immunized, and to reject any animal showing an unusually high titre of so-called normal Vi or O agglutinins. In one of the routine examinations of typhoid vaccines carried out during the last war this rule was, however, not observed, owing to the exigencies of the war situation, and the sera taken from the rabbits before and after immunization were all examined at the same time. Table 2 summarizes the results obtained in two out of several groups of rabbits that had been immunized with different batches of alcohol-treated T.A.B.C. vaccine.

Table 2. *Typhoid Vi- and O-agglutinin responses in rabbits immunized with two different batches of alcoholized T.A.B.C. vaccine*

		Rabbits immunized intravenously with									
		Alcoholized T.A.B.C. vaccine; batch A, 4 years old, Lister Institute, 1940					Alcoholized T.A.B.C. vaccine; batch B, 1 year old, Lister Institute, 1943				
	Specimen of serum	1	2	3	4	5	6	7	8	9	10
Standard TVi-agglutinin titre	I	0	0	0	20	30	0	0	10	30	20
	II	40	5	20	20	30	200	40	10	30	20
	III	80	40	200	20	30	400	80	10	30	20
Standard TO-agglutinin titre	I	0	0	0	0	0	0	0	0	0	0
	II	2,000	5,000	10,000	2,000	5,000	20,000	2,000	2,000	2,000	5,000
	III	10,000	5,000	20,000	5,000	5,000	20,000	5,000	10,000	5,000	5,000

'Standard' titres are based on the 'Provisional Standard Anti-typhoid Serum' (Felix, 1938). TVi-agglutinin titre O = a negative result in a dilution 1 in 10. TO-agglutinin titre O = a negative result in a dilution 1 in 500. I = serum taken before immunization. II = serum taken 7 days after first intravenous dose of vaccine (equivalent to 500×10^6 *Salm. typhi*). III = serum taken 7 days after second intravenous dose of vaccine (equivalent to 1000×10^6 *Salm. typhi*). For details see Felix & Anderson (1951*a*).

Table 2 shows that all the rabbits whose serum prior to immunization contained Vi agglutinins below the limit of 'normal' agglutinins, i.e. below a 'standard' titre of 1 in 10, responded to the two vaccines with abundant Vi-antibody formation. On the other hand, there was no response at all in those rabbits whose serum, before commencement of the inoculations, had a 'standard' Vi-agglutination titre of 1 in 10, or higher.

This result came as something of a surprise. In the preparation of high-titre antitoxic sera it has been the general experience that the higher the titre of normal antibody in a given animal the better is its response to immunization. Conditions

https://doi.org/10.1017/S0022172400044156 Published online by Cambridge University Press

are different with antibacterial antisera. It is known from earlier work that there is no constant relationship between the titres of the natural Vi and O antibodies in the serum of normal rabbits or horses and the subsequent immunity responses (Felix & Petrie, 1938). But the finding, illustrated in Table 2, that the presence in the serum of pre-formed Vi antibody suppressed the elaboration of antibody in response to immunization was quite unexpected. It was evident from the significant Vi-agglutinin responses in the other rabbits in the two groups that the two vaccines contained Vi antigen in agglutinogenically active form. The question of differences in the antibody-producing capacity of individual animals also did not arise, in view of the uniformly high O-agglutinin titres in the rabbits.

All the 'normal' rabbits that had unusually high Vi-agglutinin titres were from one stock, and it was soon found that the faeces of several of these animals yielded on culture a strain of *Bacterium coli* that contained the typhoid Vi antigen. Kauffmann (1941*b*) first described a coliform organism which shared with the typhoid bacillus the Vi antigen but none of its O or H antigens. Longfellow & Luippold (1943) and Luippold (1946) showed that Kauffmann's Vi-positive strain of *Bact. coli* induced in the mouse a high degree of protection against an O + Vi strain of *Salm. typhi*.

Bact. coli strains of this kind are apparently not very rare (Marmion, 1944). Rabbits (or man) harbouring such a strain may show a raised titre of TVi agglutinin and consequently may be immune to infection with typhoid bacilli. Such rabbits show no response to immunization with typhoid Vi antigen and should, therefore, not be employed in the testing of typhoid vaccine.

STANDARDIZATION OF THE POTENCY OF TYPHOID VACCINE

The desirability of standardizing the potency of typhoid vaccine by adopting an internationally agreed method of test was recognized long ago. In 1938 Prof. Th. Madsen, on behalf of the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations, invited the present writer to prepare recommendations for the introduction of a standard antityphoid vaccine and a standard method of testing its potency. The intention was to carry out comparative tests in various countries at the same time and at the same Institutes that were to participate in the international experiment on the standardization of therapeutic antityphoid serum (Report of Permanent Commission on Biological Standardization, 1938; Felix, 1938).

Contrary to the opinion held by several of the members of the Permanent Commission on Biological Standardization, the present writer felt that the plan was premature and advised against it. The main reason for this view was that no method existed at that time that would enable a 'standard vaccine' to be prepared, in which the Vi antigen could be preserved in an immunogenically active state over a reasonably long period of time. It appeared to be an indispensable condition that the 'standard' be a substance that could be dried and preserved in the manner known from the standardization of therapeutic immune sera. The only possible solution of the problem seemed to be to accept the dried 'Provisional Standard Anti-typhoid Serum' as the basis for standardizing typhoid vaccines and to employ

it in combination with one of the so-called purified antigenic fractions of the typhoid bacillus, which were also available in dried and stable form.

Three different methods of preparing these antigenic fractions had been described, namely: extraction with trichloracetic acid followed by alcohol precipitation (Boivin & Mesrobeanu, 1933), treatment with acetone followed by tryptic digestion and alcohol precipitation (Raistrick & Topley, 1934) and extraction with anhydrous diethylene-glycol followed by precipitation with acetone or alcohol (Morgan, 1937). The fraction prepared by the method of Raistrick & Topley was known to induce formation of Vi antibody of full functional efficacy (Felix & Petrie, 1938). The fraction prepared by Morgan's method already was known to contain a modified Vi antigen which stimulates production of Vi antibody deficient in protective power (Henderson & Morgan, 1938), while Boivin & Mesrobeanu's fraction had not yet been examined in this respect. It did not appear to be practicable to adopt any one of these chemically extracted antigens as 'standard' and the plan of standardizing typhoid vaccine was, for the time being, abandoned.

The position has changed since the introduction of the alcohol-killed and alcohol-preserved typhoid vaccine. It was stated already in the first paper describing the new type of vaccine that it still contained the Vi antigen in its effective form 'after storage in the cold for at least nine months, and probably much longer' (Felix, 1941). This was confirmed by tests carried out at long intervals during the war of 1939–45. It has now been found that a vaccine preserved with 25% alcohol in physiological saline and stored at 1–2° C. for 10 years does not show any loss in immunogenic potency that can be detected by passive-protection tests in mice (Felix & Anderson, 1951*a*). This would appear to be a reasonably long period of time for a vaccine to serve as 'standard'.

In addition, there is now available the alternative of using as 'standard' a dried vaccine prepared in one of the several ways that have been suggested during the past few years. Rainsford (1942) found that typhoid bacilli killed with merthiolate or alcohol, and dried with acetone, showed no loss of their Vi antibody-stimulating properties when stored in the dry state for one year at 23–25° C., or after 3 months at 37° C. Henderson, Peacock & Richley (1951) showed that acetone-dried bacterial cells of the O + Vi strain Ty 2 and of the 'pure' Vi variant Ty 6S had preserved their protective value after storage at room temperature for a period of about 10 years. Henderson, Amies & Steabben (1940) already had successfully employed acetone-dried bacilli for the preparation in the horse of therapeutic anti-typhoid serum of high Vi and O titres. Raistrick & Topley (1934) first used preparations of dried bacterial cells, killed and partially extracted with acetone, in their work on immunizing fractions derived from *Salm. typhi-murium*. When the method was applied to Vi-positive strains of the typhoid bacillus (Henderson & Morgan, 1938), the treatment was carried out with ice-cold acetone.

From the brief review given in the preceding paragraphs it is clear that what seemed to be an impossible task in 1938 has now become feasible. Either an alcoholized or a dried vaccine can now be adopted as 'standard vaccine'. This is not the place to make detailed recommendations for the standardization of the

potency of typhoid vaccines, but an outline of the suggested procedures may serve a useful purpose as a basis for discussion.

(*a*) *The routine examination in the course of preparation of typhoid vaccine*

(1) *Routine examination of the vaccine strain.* Provided the method that has been adopted for making the vaccine is strictly adhered to, the immunizing potency of the final product is determined by the Vi- and O-antigen content of the cultures employed. Uniform potency of the product is, therefore, assured by careful quantitative estimation of these two antigens in the cultures of the vaccine strain each time a batch of vaccine is made.

The methods used for the quantitative estimation of the Vi- and O-antigen content of the cultures have been described in detail (Felix, 1938; Felix & Pitt, 1951). As a rule, the routine examination consists of agglutination tests alone, using pure O and pure Vi sera. Occasionally the precaution is taken to check the results by virulence tests in the mouse or by strictly quantitative agglutinin-absorption tests. The degree of accuracy in the readings of agglutination and agglutinin-absorption tests is $\pm 20\%$.

(2) *Routine examination of the final product.* If a careful examination of the vaccine strain, as described in paragraph 1, is carried out, without exception, each time a batch of vaccine is made, there is no need for testing the immunizing potency of the final product. Occasionally samples of vaccine that have been stored at 1–2° C. for various periods of time are tested by immunizing groups of not less than five rabbits and estimating their Vi- and O-antibody responses. The 'standard vaccine' is included in each test. The agglutinin titres can be accepted as the final measure of the protective value of the Vi antigen as well as the O antigen, provided it is known that the method used for making the vaccine does not cause the peculiar modification of the Vi antigen that leads to 'functional deficiency' of the Vi antibody. It is thus unnecessary routinely to carry out passive-protection tests in mice.

(3) *Routine examination of the paratyphoid components of T.A.B.C. vaccine.* This is carried out in a manner analogous to that followed in regard to the typhoid component (Felix & Pitt, 1936; Felix & Pitt, to be published).

(*b*) *The official control of the potency of typhoid vaccine*

The position is quite different when the protective value of vaccines from various sources is estimated for purposes of official control. In this case it is clearly indispensable to employ all the available tests, irrespective of whether or not it is known by which method any given vaccine has been prepared. These tests are:

(1) Active immunization of mice.

(2) Immunization of groups of rabbits with a view to establishing:

  (*a*) the amount of circulating Vi and O antibodies elaborated;

  (*b*) whether the Vi antibody produced possesses full 'functional efficacy' in passive-protection tests in mice.

In each of these tests the 'standard vaccine' is used simultaneously with the assay of vaccines of unknown potency. In the passive-protection tests in mice

(under 2*b*) the 'Provisional Standard Anti-typhoid Serum' is also included and forms the basis of the measurement of the 'functional efficacy' of the Vi antibody. These tests are applicable to the assay of vaccines containing whole bacterial cells and of preparations of partly purified antigenic fractions.

No chemical or physical treatment of the O antigen has yet been described that would lead to the elaboration by the immunized animal or man of an O antibody of 'functional deficiency', as indicated by a decrease in the ratio of the endotoxin-neutralizing value to the O-agglutinin titre. Should, however, some form of treatment become known to cause such modification of the O antigen, it would be necessary for purposes of official control also to carry out the endotoxin-neutralization test in the mouse (Felix, 1938). In this test, too, the 'Provisional Standard Anti-typhoid Serum' could serve as the basis of assay.

What has been said about the typhoid component of T.A.B.C. vaccine also applies in analogous manner to its paratyphoid components. Separate standard sera for paratyphoid A, B and C, containing the corresponding Vi and O antibodies of each of these organisms, would have to be adopted.

DISCUSSION

The observations recorded in this paper traverse those published by a number of workers, including those in the United States of America. The discordance is obviously due to failure to pay due attention to the fundamental fact that the course of typhoid infection and the defence against it depend on the interaction between two pairs of antigens and antibodies, each of which plays a different part in the process. It has been shown in this paper how disregard of this fact in the design of mouse-protection tests inevitably obscures the results.

The degree of virulence of the culture from which the challenge dose is prepared is the most decisive factor influencing the outcome of these tests. It is well known how difficult it is to maintain laboratory strains of the typhoid bacillus at the maximum level of mouse virulence and resistance to O antibody.

The habit of using the mucin technique in order to avoid an alleged toxic effect of the relatively large challenge dose has introduced another important source of error in active- and passive-immunity experiments with the typhoid bacillus. The misleading effect of this technique has been brought out in striking manner by the experiments published by successive workers from the United States Army Medical Department [Washington]. Longfellow & Luippold (1943) and Luippold (1946), working with *Salm. ballerup* and with Kauffmann's Vi-positive strain of *Bact. coli*, concluded that the Vi antigen is of primary importance in protection against an O + Vi strain of *Salm. typhi*. Luippold (1946) and Holt (1948) even contemplated the possibility of fortifying typhoid vaccine with extracts of the Vi-positive strain of *Bact. coli*. In these experiments with heterologous vaccines the mucin technique did not cause any confusion because the protective action could only be attributed to the Vi antigen, the single antigenic component which *Salm. ballerup* and the particular strain of *Bact. coli* possess in common with *Salm. typhi*. Batson *et al.* (1950*b*), on the other hand, were unable to establish an association between the presence of the Vi antigen and the degree of protection of the vaccine, because they

experimented with variants of *Salm. typhi* which contained varying quantities of both the O and Vi antigens. Here the mucin technique precluded the demonstration of the separate effects of the two antigens. It has been stated already that the case for abandoning the use of mucin in the assay of typhoid vaccines appears to have been well established.

Griffits (1944) and Luippold (1945) attempted to standardize the method of determining the potency of typhoid vaccine. Griffits (1944) recognized that no stable standard vaccine existed at that time and attempted to control the various factors known to influence the results of potency tests. Luippold (1945) made a similar suggestion and proposed in addition the use, as a reference point, of a 'typhoid immunogenic unit' based on a particular batch of monovalent typhoid vaccine. Since this vaccine was heat-killed and phenol-preserved it could not be regarded as a stable 'standard', and the Vi antigen it contained was, of course, partly inactivated from the outset. Moreover, the tests suggested by both Griffits (1944) and Luippold (1945) were based on the use of the mucin technique and were subject to the operation of all the sources of error that have been enumerated in this paper.

Standardization of the materials and methods employed in the assay of typhoid vaccines has now become feasible. Alcoholized vaccine has been shown to remain stable for at least ten years when stored in the cold (Felix & Anderson, 1951*a*) and could serve as 'standard vaccine', to be used in combination with the 'Provisional Standard Anti-typhoid Serum'. Alternatively, a dried vaccine could be adopted as 'standard vaccine' (Rainsford, 1942; Henderson *et al.* 1951), though it is more difficult to prepare and more complicated to handle in routine tests.

SUMMARY

1. The review of the laboratory evidence published since the introduction, ten years ago, of the alcohol-treated typhoid vaccine furnishes additional support for abandoning the old method of making typhoid vaccine. The alcohol-treated vaccine has advantages in regard to both the Vi-antibody response and the degree of systemic reactions produced.

2. The technique of the preparation of alcohol-killed and alcohol-preserved typhoid-paratyphoid vaccine has remained as originally described. The methods of selecting the vaccine strains and routinely examining the cultures have also remained unchanged.

3. The necessity of testing typhoid vaccines not only by active-immunity tests in mice but also for their antibody-stimulating properties is again stressed. These tests consist of immunization of rabbits, estimation of their Vi- and O-agglutinin titres and passive-immunity tests in mice.

4. The most important sources of error in mouse-protection tests are:

(*a*) the use of a test culture of less than the maximum degree of mouse-virulence;

(*b*) the use of the mucin technique;

(*c*) immunization by intraperitoneal injections when the challenge dose also is given by this route.

Much of the experimental work on typhoid vaccine has been invalidated by these three pitfalls.

5. It is suggested that the mucin technique be abandoned in the assay of typhoid vaccine.

6. A source of error in the rabbit test is the presence of pre-formed Vi agglutinins. These are found in rabbits harbouring coliform organisms which possess the typhoid Vi antigen. Such rabbits do not respond to injections of typhoid Vi antigen.

7. Standardization of the potency of typhoid vaccine has now become possible. Either an alcohol-preserved or a dried vaccine can serve as 'standard vaccine', to be used in combination with the 'Provisional Standard Anti-typhoid Serum'. Both of these vaccines remain stable for a number of years.

8. Suggestions are briefly outlined for:

(*a*) the routine examination in the course of preparation of the vaccine;

(*b*) the official control of the potency of typhoid vaccine.

9. The paratyphoid components of T.A.B.C. vaccine can be standardized in an analogous manner.

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(*MS. received for publication* 21. IV. 51.)